PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/68

A1

(11) International Publication Number: **WO 99/42616**

(43) International Publication Date: 26 August 1999 (26.08.99)

(21) International Application Number: PCT/US99/03208

(22) International Filing Date: 15 February 1999 (15.02.99)

(30) Priority Data:
09/026,767 20 February 1998 (20.02.98) US

(71) Applicant: DADE BEHRING INC. [US/US]; 1717 Deerfield Road, Deerfield, IL 60015 (US).

(72) Inventors: PATEL, Rajesh; 37270 Northwind Terrace, Fremont, CA 94555 (US). POLLNER, Reinhold; 6376 Benzo Drive, San Jose, CA 95123 (US). ULLMAN, Edwin, F.; 135 Selby Lane, Atherton, CA 94027 (US).

(74) Agents: RUSZALA, Lois, K. et al.; Dade Behring Inc., 1717 Deerfield Road, Deerfield, IL 60015 (US).

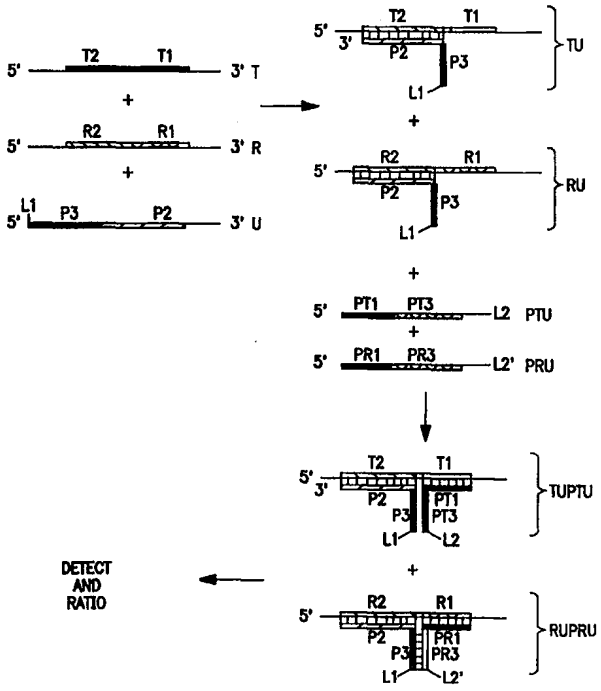
(81) Designated States: CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHODS FOR DETERMINING CONCENTRATIONS OF NUCLEIC ACIDS

(57) Abstract

The present invention relates to a method for detecting a single stranded target polynucleotide. A ternary complex is formed comprising the target polynucleotide and first and second oligonucleotide probes, which respectively have different first sequences that are complementary to the target polynucleotide and different second sequences that are complementary to each other. The complex is formed under conditions wherein the first and second oligonucleotide probes do not substantially bind each other in the absence of the target polynucleotide. The association of the first and second oligonucleotide probes is then detected. In another embodiment of the present invention a first ternary complex is formed as above and a second ternary complex is formed comprising a reference polynucleotide and the first oligonucleotide probe and a third oligonucleotide probe. The reference polynucleotide has a sequence that is complementary to the first sequence of the first oligonucleotide probe. The third oligonucleotide probe has a first sequence that is complementary to the reference polynucleotide but not to the target polynucleotide and a second sequence that is identical to the second sequence of the second oligonucleotide probe. The conditions for forming the ternary complexes are controlled to avoid substantial binding of the first oligonucleotide probe with the second and third oligonucleotide probes in the absence of the target and reference polynucleotides. The ratio of the ternary complexes is determined and related to the amount of the target polynucleotide. Kits for carrying out the above methods are also disclosed.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

METHODS FOR DETERMINING CONCENTRATIONS OF NUCLEIC ACIDS

BACKGROUND OF THE INVENTION

1. Field of the Invention.

Significant morbidity and mortality are associated with infectious diseases. More rapid and accurate diagnostic methods are required for better monitoring and treatment of disease. Molecular methods using DNA probes, nucleic acid hybridizations and in vitro amplification techniques are promising methods offering advantages to conventional methods used for patient diagnoses.

Nucleic acid hybridization has been employed for investigating the identity and establishing the presence of nucleic acids. Hybridization is based on complementary base pairing. When complementary single stranded nucleic acids are incubated together, the complementary base sequences pair to form double stranded hybrid molecules. The ability of single stranded deoxyribonucleic acid (ssDNA) or ribonucleic acid (RNA) to form a hydrogen bonded structure with a complementary nucleic acid sequence has been employed as an analytical tool in molecular biology research. The availability of radioactive nucleoside triphosphates of high specific activity and the ^{32}P labeling of DNA with T4 polynucleotide kinase has made it possible to identify, isolate, and characterize various nucleic acid sequences of biological interest. Nucleic acid hybridization has great potential in diagnosing disease states associated with unique nucleic acid sequences. These unique nucleic acid sequences may result from genetic or environmental change in DNA by insertions, deletions, point mutations, or by acquiring foreign DNA or RNA by means of infection by bacteria, molds, fungi, and viruses. Nucleic acid hybridization has, until now, been employed primarily in academic and industrial molecular biology laboratories. The application of nucleic acid hybridization as a diagnostic tool in clinical medicine is limited because of the frequently very low concentrations of disease related DNA or RNA present in a patient's body fluid and the unavailability of a sufficiently sensitive method of nucleic acid hybridization analysis.

One method for detecting specific nucleic acid sequences generally involves immobilization of the target nucleic acid on a solid support such as nitrocellulose paper, cellulose paper, diazotized paper, or a nylon membrane. After the target nucleic acid is fixed on the support, the support is contacted with a suitably labeled probe nucleic acid for about two to forty-eight hours. After the above time period, the solid support is washed several times at a controlled temperature to remove unhybridized probe. The support is then dried and the hybridized material is detected by autoradiography or by spectrometric methods.

When very low concentrations must be detected, the above method is slow and labor intensive, and nonisotopic labels that are less readily detected than radiolabels are frequently not suitable.

Various methods have been developed for amplifying the number of molecules of DNA or RNA in a sample, which may then be analyzed. One method for the enzymatic amplification of specific segments of DNA known as the polymerase chain reaction (PCR) method has been described. This in vitro amplification procedure is based on repeated cycles of denaturation, oligonucleotide primer annealing, and primer extension by thermophilic polymerase, resulting in the exponential increase in copies of the region flanked by the primers. The PCR primers, which anneal to opposite strands of the DNA, are positioned so that the polymerase catalyzed extension product of one primer can serve as a template strand for the other, leading to the accumulation of a discrete fragment whose length is defined by the distance between the 5' ends of the oligonucleotide primers.

Other methods for amplifying nucleic acids are single primer amplification, ligase chain reaction (LCR), nucleic acid sequence based amplification (NASBA) and the Q-beta-replicase method. Regardless of the amplification used, the amplified product must be detected.

One method for detecting nucleic acids is to employ nucleic acid probes that bind to the target polynucleotide. A means for detecting such binding is also employed. Numerous configurations have been devised although only a few are suitable for homogenous detection, that is, detection by mixing the sample with reagent and then reading a signal without a separation step. One class of

homogeneous methods relies on the formation of a sandwich comprising two probes and the target. For example, Heller discloses the use of two probes that bind near each other, one having a fluorescent energy donor such a fluorescein and the other an energy acceptor such as rhodamine. Binding causes a change in the fluorescence intensity and/or wavelength. Methods have also been devised for signaling the binding of a single probe to the target. GenProbe uses a probe bound to a chemiluminescent acridinium ester that provides a signal that is modulated as a result of binding. Coates (J. Chem. Soc., Chem. Comm. (1995) __:1107) uses a europium labeled probe that becomes fluorescent when binding occurs. Hoffmann La Roche uses a probe in their Taqman technology that is labeled with both a fluorescent energy donor and an energy acceptor. An enzyme cleaves the probe only when it is bound to the target. The separated labels have increased fluorescence.

All of the currently available homogeneous detection methods have limited sensitivity, either because the signal that can be produced is relatively weak or because of high background signal derived from unbound probe. Additionally, none of these methods is particularly satisfactory for quantitation of the amount of target. Sandwich methods suffer from the so-called "prozone phenomenon" in which the signal is observed to increase with increasing target concentration up to a point but on further increases in concentration the signal drops off. Single probe methods also lose their ability to quantitate at high target concentration because the amount of probe that becomes bound under these conditions cannot exceed the amount of probe used, and, therefore, the amount is not proportional to the amount of target.

It is desirable to have a sensitive, simple method for amplifying and detecting nucleic acids, preferably, in a homogeneous format. The method should minimize the number and complexity of steps and reagents. Furthermore, the method should permit quantitative detection of a target nucleic acid regardless of its concentration.

2. Description of the Related Art

Heller in U.S. Patent No. 5,565,322 discloses hybridization of polynucleotides conjugated with chromophores and fluorophores to generate donor to donor energy transfer system.

Rapid, non-separation electrochemiluminescent DNA hybridization assays for PCR products using 3'-labeled oligonucleotide probes is described by Gudibande, et al., (1992) Molecular and Cellular Probes, 6: 495-503. A related disclosure is found in international patent application WO 9508644 A1 (950330).

Marmaro, et al., (Meeting of the American Association of Clinical Chemists, San Diego, California, November 1994, Poster No. 54) discusses the design and use of fluorogenic probes in TaqMan, a homogeneous PCR assay.

A PCR-based assay that utilizes the inherent 5' nuclease of rTth DNA polymerase for the quantitative detection of HCV RNA is disclosed by Tsang, et al., (94th General Meeting of the American Society for Microbiology, Las Vegas NE 5/94, Poster No. C376).

German patent application DE 4234086-A1 (92.02.05) (Henco, et al.) discusses the determination of nucleic acid sequences amplified in vitro in enclosed reaction zone where probe(s) capable of interacting with target sequence is present during or after amplification and spectroscopically measurable parameters of probe undergo change thereby generating signal.

U.S. Patent No. 5,232,829 (Longiaru, et al.) discloses detection of Chlamydia trachomatis by polymerase chain reaction using biotin labeled DNA primers and capture probes. A similar disclosure is made by Loeffelholz, et al. (1992) Journal of Clinical Microbiology, 30(11):2847-2851.

Padlock probes: circularizing oligonucleotides for localized DNA detection are described by Nilsson, et al. (1994) Science, 265:2085-2088.

A process for amplifying, detecting and/or cloning nucleic acid sequences is disclosed in U.S. Patent Nos. 4,683,195, 4,683,202, 4,800,159, 4,965,188 and 5,008,182. Sequence polymerization by polymerase chain reaction is described by Saiki, et al., (1986) Science, 230: 1350-1354. Primer-directed enzymatic

amplification of DNA with a thermostable DNA polymerase is described by Saiki, et al., Science (1988) 239:487.

U.S. Patent Applications Serial Nos. 07/299,282 and 07/399,795, filed January 19, 1989, and August 29, 1989, respectively, now U.S. Patent No. 5,508,178, describe nucleic acid amplification using a single polynucleotide primer (ASPP). A method for introducing defined sequences at the 3'-end of a polynucleotide is described in U.S. Patent Application Serial No. 08/140,369, filed October 20, 1993, now U.S. Patent No. 5,679,512. The disclosures of these applications are incorporated herein by reference including the references listed therein in the sections entitled "Description of the Related Art."

SUMMARY OF THE INVENTION

One embodiment of the present invention is a method for detecting a single stranded target polynucleotide. A ternary complex is formed comprising the target polynucleotide and first and second oligonucleotide probes, which, respectively, have different first sequences that are complementary to the target polynucleotide and different second sequences that are complementary to each other. The complex is formed under conditions wherein the first and second oligonucleotide probes do not substantially bind to each other in the absence of the target polynucleotide. The association of the first and second oligonucleotide probes is then detected.

Another embodiment of the present invention is a method for determining the amount of a target polynucleotide. A first ternary complex is formed comprising the target polynucleotide and first and second oligonucleotide probes wherein the first and second oligonucleotide probes respectively have different first sequences that are complementary to the target polynucleotide and different second sequences that are complementary to each other. A second ternary complex is formed comprising a reference polynucleotide and the first oligonucleotide probe and a third oligonucleotide probe. The reference polynucleotide has a sequence that is complementary to the first sequence of the first oligonucleotide probe. The third oligonucleotide probe has a first sequence that is complementary to the reference

polynucleotide but not to the target polynucleotide and a second sequence that is identical to the second sequence of the second oligonucleotide probe. The conditions for forming the ternary complexes are controlled to avoid substantial binding of the first oligonucleotide probe with the second and third oligonucleotide probes in the absence of the target and reference polynucleotide probes. The ratio of the ternary complexes is determined and related to the amount of the target polynucleotide.

Another embodiment of the present invention is a method for detecting a single stranded target polynucleotide. A combination is provided comprising a medium suspected of containing the target polynucleotide having a sequence T2. Also included in the combination is a reference polynucleotide having a sequence R2 that is identical to T2 and a sequence R1 that is not present in the target polynucleotide. The combination further comprises a first oligonucleotide probe capable of independently hybridizing to T2 and R2, a second oligonucleotide probe that binds to a duplex TU of the target polynucleotide and the first oligonucleotide probe but not to the target polynucleotide or the first oligonucleotide probe apart from TU, and a third oligonucleotide probe that binds to a duplex RU of the reference polynucleotide and the first oligonucleotide probe but not to the reference polynucleotide or the first oligonucleotide probe apart from RU. The combination is incubated under conditions such that the target polynucleotide and the reference polynucleotide can bind, respectively, to the first oligonucleotide probe to form TU and RU and the second and third oligonucleotide probes bind respectively to TU and RU to form ternary complexes. A determination of the ratio of the ternary complexes is made and related to the concentration of the target polynucleotide in the medium.

Another aspect of the present invention is a method for determining the amount of a single stranded target polynucleotide present in a medium. A combination is provided comprising a measured amount of a medium suspected of containing the single stranded target polynucleotide, which comprises a sequence T1 and a sequence T2. Also in the combination is a predetermined amount of a reference polynucleotide comprising a sequence R2 that is identical to T2 and a sequence R1 that is not present in the target polynucleotide. The combination

further includes a first oligonucleotide probe comprising a first sequence P2 capable of hybridizing to R2 and T2 and a sequence P3 that is not capable of hybridizing to the target polynucleotide or the reference polynucleotide, a second oligonucleotide probe comprising a sequence PT1 capable of hybridizing to T1 and a sequence PT3 that is capable of hybridizing to P3, and a third oligonucleotide probe comprising a sequence PR1 that is capable of hybridizing to R1 and a sequence PR3 that is identical to PT3 and is capable of hybridizing to P3. R1, T1, P3, PR1, PR3, PT1 and PT3 are constructed such that respective hybridizable pairs thereof do not form stable duplexes under the incubation conditions employed in the method. The combination is incubated under conditions such that the target polynucleotide and the reference polynucleotide bind, respectively, to the first oligonucleotide probe to form independent duplexes TU and RU and the second and third oligonucleotide probes bind respectively to TU and RU to form ternary complexes. The ratio of the ternary complexes is determined and related to the amount of the single stranded target polynucleotide in the medium.

Another embodiment of the present invention is a method for quantitating a target polynucleotide by amplifying and detecting an amplification product of said target polynucleotide, which is comprised of sequences T1 and T2. The method comprises: (a) providing in combination (i) a medium suspected of containing the target polynucleotide, said medium having a measured concentration of a reference polynucleotide that yields a reference amplification product comprising a sequence R2 that is identical to said sequence T2, and a sequence R1 that is different from said sequence T1, (ii) all reagents required for conducting an amplification of said target and said reference polynucleotides to produce amplification products of said target and said reference polynucleotides, (iii) a first oligonucleotide probe comprising a first sequence P2 capable of hybridizing to R2 and T2 and a sequence P3 that is not capable of hybridizing to the amplification products of said target polynucleotide or said reference polynucleotide, (iv) a second oligonucleotide probe comprising a sequence PT1 capable of hybridizing to T1 and a sequence PT3 that is capable of hybridizing to P3, and (v) a third oligonucleotide probe comprising a sequence PR1 that is capable of hybridizing to R1 and a sequence PR3 that is

identical to PT3 and is capable of hybridizing to P3, wherein sequences R1, T1, P3, PR1, PR3, PT1 and PT3 are constructed such that respective hybridizable pairs thereof do not form stable duplexes under the conditions in step (b), (b) incubating the combination under conditions such that amplification products of the target polynucleotide and the reference polynucleotide are formed and bind, respectively, to the first oligonucleotide probe to form independent duplexes TU and RU and the second and third oligonucleotide probes bind respectively to TU and RU to form ternary complexes, (c) determining the ratio of the ternary complexes, and (d) relating the ratio to the concentration of the target polynucleotide in the medium during the amplification.

Another embodiment of the present invention is a method for quantitating a target polynucleotide by amplifying and detecting an amplification product of the target RNA, which is comprised of sequences T1 and T2. A combination is provided comprising a medium suspected of containing the target RNA, said medium having a measured concentration of a reference polynucleotide that yields a reference amplification product comprising a sequence R2 that is identical to said sequence T2, and a sequence R1 that is different from sequence T1. All reagents required for conducting an amplification of the target RNA and the reference polynucleotide by NASBA to produce amplification products of the target RNA and the reference polynucleotide are included in the combination. The combination also includes a first oligonucleotide probe comprising a first sequence P2 capable of hybridizing to R2 and T2 and a sequence P3 that is not capable of hybridizing to the amplification products of said target RNA or the reference polynucleotide, a second oligonucleotide probe comprising a sequence PT1 capable of hybridizing to T1 and a sequence PT3 that is capable of hybridizing to P3, and a third oligonucleotide probe comprising a sequence PR1 that is capable of hybridizing to R1 and a sequence PR3 that is identical to PT3 and is capable of hybridizing to P3. R1, T1, P3, PR1, PR3, PT1 and PT3 are constructed such that respective hybridizable pairs thereof do not form stable duplexes under the conditions of incubation used in the method. The combination is incubated under conditions such that amplification products of the target RNA and the reference polynucleotide are formed and bind, respectively,

to the first oligonucleotide probe to form independent duplexes TU and RU and the second and third oligonucleotide probes bind respectively to TU and RU to form ternary complexes. The ratio of the ternary complexes is determined and related to the concentration of the target RNA in the medium during the amplification.

Another aspect of the present invention is a kit for detection of a single stranded target polynucleotide wherein the kit comprises in packaged combination first and second oligonucleotide probes. The probes respectively different first sequences that are both complementary to the target polynucleotide and different second sequences that are complementary to each other and 8 to 16 nucleotides in length.

Another aspect of the present invention is a kit for use in detection of a target polynucleotide comprising sequences T1 and T2. The kit comprises in packaged combination: (a) a predetermined amount of a reference polynucleotide comprising a sequence R2 that is identical to T2 and a sequence R1 that is not present in the target polynucleotide, (b) a first oligonucleotide probe comprising a first sequence P2 capable of hybridizing to R2 and T2 and a sequence P3 that is not capable of hybridizing to the target polynucleotide or the reference polynucleotide, (c) a second oligonucleotide probe comprising a sequence PT1 capable of hybridizing to T1 and a sequence PT3 that is capable of hybridizing to P3, and (d) a third oligonucleotide probe comprising a sequence PR1 that is capable of hybridizing to R1 and a sequence PR3 that is identical to PT3 and is capable of hybridizing to P3. The lengths of R1, T1, P3, PR1, PR3, PT1 and PT3 are such that respective hybridizable pairs thereof do not form stable duplexes under conditions for formation of a stable ternary complex of the target polynucleotide with the first and second oligonucleotide probes.

Another aspect of the present invention is a kit for use in quantitating a target RNA by amplifying and detecting an amplification product of the target RNA, which comprises sequences T1 and T2. The kit comprises in packaged combination: (a) a promoter, (b) an RNA polymerase, (c) a predetermined amount of a reference polynucleotide comprising a sequence R2 that is identical to T2 and a sequence R1 that is different from sequence T1, (d) a first oligonucleotide probe comprising a first

sequence P2 capable of hybridizing to R2 and T2 and a sequence P3 that is not capable of hybridizing to the target RNA or the reference polynucleotide, (e) a second oligonucleotide probe comprising a sequence PT1 capable of hybridizing to T1 and a sequence PT3 that is capable of hybridizing to P3, and (f) a third oligonucleotide probe comprising a sequence PR1 that is capable of hybridizing to R1 and a sequence PR3 that is identical to PT3 and is capable of hybridizing to P3. The lengths of R1, T1, P3, PR1, PR3, PT1 and PT3 are such that respective hybridizable pairs thereof do not form stable duplexes under the conditions for formation of a stable ternary complex of the target amplification product with the first and second oligonucleotide probes.

Another embodiment of the present invention is a reagent for detecting a target polynucleotide. The reagent comprises two oligonucleotide probes capable of binding to a single strand of the target polynucleotide. The first and second oligonucleotide probes respectively have different first sequences that are complementary to the target polynucleotide and different second sequences that are complementary to each other and 8 to 16 nucleotides in length.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a schematic diagram depicting one embodiment in accordance with the present invention.

Fig. 2 is a schematic diagram depicting an alternate embodiment in accordance with the present invention.

Fig. 3 is a schematic diagram depicting an alternate embodiment in accordance with the present invention.

Fig. 4 is a schematic diagram depicting an alternate embodiment in accordance with the present invention.

Fig. 5 is a schematic diagram depicting an alternate embodiment in accordance with the present invention.

Fig. 6 is a schematic diagram depicting the experiments described in Example 3.

Fig. 7 is a graph showing the results of the experiments described in Example 3.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

In its broadest aspect the present invention relates to a method for detecting a target polynucleotide and is particularly useful for quantitatively detecting a target polynucleotide. More particularly, the present invention relates to a method for amplifying and detecting a target polynucleotide. Thus, the present invention provides for detection of the products of nucleic acid amplification reactions. All of the necessary reagents for amplification and detection may be included in the reaction mixture prior to amplification. It is not necessary to open the reaction vessel and/or to separate reagents and products after amplification and prior to binding of probes, which binding is subsequently subjected to detection. Thus, the amplification and detection can be conducted in a homogeneous manner thus providing for, among others, ease of operation and avoidance of contamination.

One aspect of the present invention is a method based on determining the concentration of a target polynucleotide by measuring the ratio of the target polynucleotide to a reference polynucleotide of known concentration or the ratio of their amplification products. A known amount of a reference polynucleotide that has a sequence in common with the target polynucleotide is added to a measured amount of the sample that contains the target polynucleotide. Ternary complexes are formed comprising (i) the target polynucleotide and a first and a second oligonucleotide probe and (ii) the reference polynucleotide the first and a third oligonucleotide probes. The first probe has a first sequence capable of binding to both the target and the reference polynucleotides and a second sequence that is complementary to a second sequence present in the second and third oligonucleotide probes. The second and third oligonucleotide probes have first sequences that bind specifically to the target and reference oligonucleotides, respectively. The complexes are formed under conditions wherein the first oligonucleotide probe does not substantially bind to the second and third oligonucleotide probes in the absence of the target or reference polynucleotides. The association of the first oligonucleotide probe with the second and third oligonucleotide probes is then detected.

Before proceeding further with a description of the specific embodiments of the present invention, a number of terms will be defined.

Polynucleotide analyte -- a compound or composition to be measured that is a polymeric nucleotide, which in the intact natural state can have about 20 to 5,000,000 or more nucleotides and in an isolated state can have about 30 to 50,000 or more nucleotides, usually about 100 to 20,000 nucleotides, more frequently 500 to 10,000 nucleotides. It is thus obvious that isolation of the analyte from the natural state often results in fragmentation. The polynucleotide analytes include nucleic acids, and fragments thereof, from any source in purified or unpurified form including DNA (dsDNA and ssDNA) and RNA, including t-RNA, m-RNA, r-RNA, mitochondrial DNA and RNA, chloroplast DNA and RNA, DNA-RNA hybrids, or mixtures thereof, genes, chromosomes, plasmids, the genomes of biological material such as microorganisms, e.g., bacteria, yeasts, viruses, viroids, molds, fungi, plants, animals, humans, and the like. The polynucleotide analyte can be only a minor fraction of a complex mixture such as a biological sample. The analyte can be obtained from various biological materials by procedures well known in the art. Some examples of such biological material by way of illustration and not limitation are disclosed in the following Table 1:

Table 1

Microorganisms of interest include:

Corynebacteria
Corynebacterium diphtheria
Pneumococci
Diplococcus pneumoniae
Streptococci
Streptococcus pyrogenes
Streptococcus salivarus
Staphylococci
Staphylococcus aureus
Staphylococcus albus
Neisseria
Neisseria meningitidis
Neisseria gonorrhea
Enterobacteriaciae
Escherichia coli
Aerobacter aerogenes
Klebsiella pneumoniae
Salmonella typhosa
Salmonella choleraesuis
Salmonella typhimurium
Shigella dysenteria
Shigella schmitzii
Shigella arabinotarda
Shigella flexneri
Shigella boydii
Shigella sonnei
Other enteric bacilli
Proteus vulgaris
Proteus mirabilis
Proteus morgani
Pseudomonas aeruginosa
Alcaligenes faecalis
Vibrio cholerae

Hemophilus-Bordetella group
Hemophilus influenza, H. ducryi
Hemophilus hemophilus
Hemophilus aegypticus
Hemophilus parainfluenza
Bordetella pertussis
Pasteurellae

The colliform bacteria

The *Salmonellae*

The *Shigellae*

Proteus species

Rhizopus oryzae
Rhizopus arrhizua Phycomycetes
Rhizopus nigricans
Sporotrichum schenkii
Flonsecaea pedrosoi
Fonsecacea compact
Fonsecacea dermatidis

Pasteurella pestis
Pasteurella tulareusis
Brucellae
Brucella melitensis
Brucella abortus
Brucella suis

Aerobic Spore-forming Bacilli
Bacillus anthracis
Bacillus subtilis
Bacillus megaterium
Bacillus cereus
Anaerobic Spore-forming Bacilli
Clostridium botulinum
Clostridium tetani
Clostridium perfringens
Clostridium novyi
Clostridium septicum
Clostridium histolyticum
Clostridium tertium
Clostridium bifermentans
Clostridium sporogenes
Mycobacteria
Mycobacterium tuberculosis hominis
Mycobacterium bovis
Mycobacterium avium
Mycobacterium leprae
Mycobacterium paratuberculosis
Actinomycetes (fungus-like bacteria)
Actinomyces Isaeli
Actinomyces bovis
Actinomyces naeslundii
Nocardia asteroides
Nocardia brasiliensis
The Spirochetes
Treponema pallidum Spirillum minus
Treponema pertenue Streptobacillus monoiliformis
Treponema carateum
Borrelia recurrentis
Leptospira icterohemorrhagiae
Leptospira canicola
Trypanasomes
Mycoplasmas
Mycoplasma pneumoniae

Cladosporium carrionii
Phialophora verrucosa
Aspergillus nidulans
Madurella mycetomi
Madurella grisea
Allescheria boydii

Phialophora jeanselmei
Microsporum gypseum
Trichophyton mentagrophytes
Keratinomyces ajelloi
Microsporum canis
Trichophyton rubrum
Microsporum adouini
Viruses
Adenoviruses
Herpes Viruses
Herpes simplex
Varicella (Chicken pox)
Herpes Zoster (Shingles)
Virus B
Cytomegalovirus
Pox Viruses
Variola (smallpox)

Vaccinia
Poxvirus bovis
Paravaccinia
Molluscum contagiosum
Picornaviruses
Poliovirus
Coxsackievirus
Echoviruses
Rhinoviruses
Myxoviruses
Influenza (A, B, and C)
Parainfluenza (1-4)
Mumps Virus
Newcastle Disease Virus
Measles Virus
Rinderpest Virus
Canine Distemper Virus
Respiratory Syncytial Virus
Rubella Virus
Arboviruses

Other pathogens
Listeria monocytogenes
Erysipelothrix rhusiopathiae
Streptobacillus moniliformis
Donvania granulomatis
Bartonella bacilliformis
Rickettsiae (bacteria-like parasites)
Rickettsia prowazekii
Rickettsia mooseri
Rickettsia rickettsii
Rickettsia conori
Rickettsia australis
Rickettsia sibiricus
Rickettsia akari
Rickettsia tsutsugamushi
Rickettsia burnetti
Rickettsia quintana
Chlamydia (unclassifiable parasites bacterial/viral)
Chlamydia agents (naming uncertain)
Fungi
Cryptococcus neoformans
Blastomyces dermatidis
Hisoplasma capsulatum
Coccidioides immitis
Paracoccidioides brasiliensis
Candida albicans
Aspergillus fumigatus
Mucor corymbifer (Absidia corymbifera)
Eastern Equine Eucephalitis Virus
Western Equine Eucephalitis Virus
Sindbis Virus
Chikugunya Virus
Semliki Forest Virus
Mayora Virus
St. Louis Encephalitis Virus
California Encephalitis Virus
Colorado Tick Fever Virus
Yellow Fever Virus
Dengue Virus
Reoviruses
Reovirus Types 1-3
Retroviruses
Human Immunodeficiency Viruses (HIV)
Human T-cell Lymphotrophic Virus I & II (HTLV)
Hepatitis
Hepatitis A Virus
Hepatitis B Virus
Hepatitis nonA-nonB Virus
Tumor Viruses
Rauscher Leukemia Virus
Gross Virus
Maloney Leukemia Virus
Human Papilloma Virus

Also included are genes, such as hemoglobin gene for sickle-cell anemia, cystic fibrosis gene, oncogenes, cDNA, and the like.

The polynucleotide analyte, where appropriate, may be cleaved to obtain a fragment that contains a target polynucleotide sequence, for example, by shearing or by treatment with a restriction endonuclease or other site specific chemical cleavage method.

For purposes of this invention, the polynucleotide analyte, or a cleaved fragment obtained from the polynucleotide analyte, will usually be at least partially denatured or single stranded or treated to render it denatured or single stranded.

Such treatments are well known in the art and include, for instance, heat or alkali treatment. For example, double stranded DNA can be heated at 90-100° C. for a period of about 1 to 10 minutes to produce denatured material.

Amplification of nucleic acids or polynucleotides -- any method that results in the formation of multiple copies of a nucleic acid or polynucleotide molecule or in the formation of multiple copies of the complement of a nucleic acid or polynucleotide molecule.

Exponential amplification of nucleic acids or polynucleotides -- any method that depends on the product catalyzed formation of multiple copies of a nucleic acid or polynucleotide molecule or its complement. The amplification products are sometimes referred to as "amplicons."

As mentioned above, one such method for the enzymatic amplification of specific double stranded sequences of DNA is known as the polymerase chain reaction (PCR), as described above. This in vitro amplification procedure is based on repeated cycles of denaturation, oligonucleotide primer annealing, and primer extension by thermophilic template dependent polynucleotide polymerase, resulting in the exponential increase in copies of the desired sequence of the polynucleotide analyte flanked by the primers. The two different PCR primers, which anneal to opposite strands of the DNA, are positioned so that the polymerase catalyzed extension product of one primer can serve as a template strand for the other, leading to the accumulation of a discrete double stranded fragment whose length is defined by the distance between the 5' ends of the oligonucleotide primers. The reagents for conducting such an amplification include the oligonucleotide primers, a nucleotide polymerase and nucleoside triphosphates such as, e.g., deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxycytidine triphosphate (dCTP) and deoxythymidine triphosphate (dTTP).

Another method for amplification is mentioned above and involves amplification of a single stranded polynucleotide using a single oligonucleotide primer. The single stranded polynucleotide that is to be amplified contains two non-contiguous sequences that are complementary to one another and, thus, are capable of hybridizing together to form a stem-loop structure. This single stranded

polynucleotide already may be part of a polynucleotide analyte or may be created as the result of the presence of a polynucleotide analyte. The reagents for conducting such an amplification include single oligonucleotide primer, a nucleotide polymerase and nucleoside triphosphates such as, e.g., deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxycytidine triphosphate (dCTP) and deoxythymidine triphosphate (dTTP).

Another method for achieving the result of an amplification of nucleic acids is known as the ligase chain reaction (LCR). This method uses as reagents a ligase enzyme to join pairs of preformed nucleic acid probes. The probes hybridize with each complementary strand of the nucleic acid analyte, if present, and ligase is employed to bind each pair of probes together resulting in two templates that can serve in the next cycle to reiterate the particular nucleic acid sequence.

Another method for achieving nucleic acid amplification is the nucleic acid sequence based amplification (NASBA). This method is a promoter-directed, enzymatic process that induces in vitro continuous, homogeneous and isothermal amplification of a specific nucleic acid to provide RNA copies of the nucleic acid. The reagents for conducting NASBA include a first DNA primer with a 5' tail comprising a promoter, a second DNA primer, reverse transcriptase, RNAse-H, T7 RNA polymerase, NTP's and dNTP's.

Another method for amplifying a specific group of nucleic acids is the Q-beta-replicase method, which relies on the ability of Q-beta-replicase to amplify its RNA substrate exponentially. The reagents for conducting such amplification include a "midi-variant RNA" (amplifiable hybridization probe), NTP's and Q-beta-replicase.

Another method for amplifying nucleic acids is known as 3SR and is similar to NASBA except that the RNAse-H activity is present in the reverse transcriptase.

Linear amplification of nucleic acids or polynucleotides -- any method that depends on the self-catalyzed formation of one or more copies of the complement of only one strand of a nucleic acid or polynucleotide molecule, usually a nucleic acid or polynucleotide analyte. Thus, the primary difference between linear amplification and exponential amplification is that the latter is autocatalytic, that is, the product serves to catalyze the formation of more product, whereas in the former process the starting

sequence catalyzes the formation of product but it is not itself replicated. In linear amplification the amount of product formed increases as a linear function of time as opposed to exponential amplification where the amount of product is an exponential function of time.

Target polynucleotide -- a sequence of nucleotides to be identified, usually a portion (target polynucleotide) or all of a polynucleotide analyte, the identity of which is known to an extent sufficient to allow preparation of various oligonucleotides such as probes and primers and other molecules necessary for conducting an amplification and detection of the target polynucleotide.

In general, in amplification procedures involving primer extension, amplification primers hybridize to, and are extended along (chain extended), at least the target sequence within the target polynucleotide and, thus, the target sequence acts as a template. The extended primers are chain "extension products." The target sequence usually lies between two defined sequences but need not. In general, the primers hybridize with the defined sequences or with at least a portion of such target polynucleotide, usually at least a ten nucleotide segment at the 3'-end thereof and preferably at least 15, frequently 20 to 50 nucleotide segment thereof. The target sequence usually contains from about 30 to 5,000 or more nucleotides, preferably 50 to 1,000 nucleotides. The target polynucleotide is generally a fraction of a larger molecule or it may be substantially the entire molecule (polynucleotide analyte). The minimum number of nucleotides in the target polynucleotide sequence is selected to assure that the presence of target polynucleotide in a sample is a specific indicator of the presence of polynucleotide analyte in a sample. Very roughly, the sequence length is usually greater than about 1.6 log L nucleotides where L is the number of base pairs in the genome of the biologic source of the sample. The maximum number of nucleotides in the target polynucleotide is normally governed by the length of the polynucleotide analyte and its tendency to be broken by shearing, or other processes during isolation and any procedures required to prepare the sample for assay and the efficiency of detection and/or amplification of the sequence.

Oligonucleotide -- a polynucleotide, usually single stranded, usually a synthetic polynucleotide but may be a naturally occurring polynucleotide. The oligonucleotide(s) are usually comprised of a sequence of at least 5 nucleotides, preferably, 10 to 100 nucleotides, more preferably, 20 to 50 nucleotides, and usually 10 to 30 nucleotides in length.

Various techniques can be employed for preparing an oligonucleotide utilized in the present invention. Such oligonucleotide can be obtained by biological synthesis or by chemical synthesis. For short sequences (up to about 100 nucleotides) chemical synthesis will frequently be more economical as compared to the biological synthesis. In addition to economy, chemical synthesis provides a convenient way of incorporating low molecular weight compounds and/or modified bases during the synthesis step. Furthermore, chemical synthesis is very flexible in the choice of length and region of the target polynucleotide binding sequence. The oligonucleotide can be synthesized by standard methods such as those used in commercial automated nucleic acid synthesizers. Chemical synthesis of DNA on a suitably modified glass or resin can result in DNA covalently attached to the surface. This may offer advantages in washing and sample handling. For longer sequences standard replication methods employed in molecular biology can be used such as the use of M13 for single stranded DNA as described by J. Messing (1983) Methods Enzymol, 101, 20-78.

Other methods of oligonucleotide synthesis include phosphotriester and phosphodiester methods (Narang, et al. (1979) Meth. Enzymol 68: 90) and synthesis on a support (Beaucage, et al. (1981) Tetrahedron Letters 22: 1859-1862) as well as phosphoramidate technique, Caruthers, M. H., et al., "Methods in Enzymology," Vol. 154, pp. 287-314 (1988), and others described in "Synthesis and Applications of DNA and RNA," S.A. Narang, editor, Academic Press, New York, 1987, and the references contained therein.

Oligonucleotide probe -- an oligonucleotide employed in the present invention to bind to a portion of a target polynucleotide or a reference polynucleotide. The design and preparation of the oligonucleotide probes are important in performing the

methods of this invention. A more detailed description of oligonucleotide probes in accordance with the present invention is found hereinbelow.

Oligonucleotide primer(s) -- an oligonucleotide that is usually employed in a chain extension on a polynucleotide template such as in, for example, an amplification of a nucleic acid. The oligonucleotide primer is usually a synthetic nucleotide that is single stranded, containing a sequence at its 3'-end that is capable of hybridizing with a defined sequence of the target polynucleotide. Normally, an oligonucleotide primer has at least 80%, preferably 90%, more preferably 95%, most preferably 100%, complementarity to a defined sequence or primer binding site. The number of nucleotides in the hybridizable sequence of an oligonucleotide primer should be such that stringency conditions used to hybridize the oligonucleotide primer will prevent excessive random non-specific hybridization. Usually, the number of nucleotides in the oligonucleotide primer will be at least as great as the defined sequence of the target polynucleotide, namely, at least ten nucleotides, preferably at least 15 nucleotides and generally from about 10 to 200, preferably 20 to 50, nucleotides.

Nucleoside triphosphates -- nucleosides having a 5'-triphosphate substituent. The nucleosides are pentose sugar derivatives of nitrogenous bases of either purine or pyrimidine derivation, covalently bonded to the 1'-carbon of the pentose sugar, which is usually a deoxyribose or a ribose. The purine bases include adenine (A), guanine (G), inosine (I), and derivatives and analogs thereof. The pyrimidine bases include cytosine (C), thymine (T), uracil (U), and derivatives and analogs thereof. Nucleoside triphosphates include deoxyribonucleoside triphosphates such as the four common triphosphates dATP, dCTP, dGTP and dTTP and ribonucleoside triphosphates such as the four common triphosphates rATP, rCTP, rGTP and rUTP.

The term "nucleoside triphosphates" also includes derivatives and analogs thereof, which are exemplified by those derivatives that are recognized in a similar manner to the underivatized nucleoside triphosphates. Examples of such derivatives or analogs, by way of illustration and not limitation, are those which are biotinylated, amine modified, alkylated, and the like and also include phosphorothioate, phosphite, ring atom modified derivatives, and the like.

Nucleotide -- a base-sugar-phosphate combination that is the monomeric unit of nucleic acid polymers, i.e., DNA and RNA.

Modified nucleotide -- is the unit in a nucleic acid polymer that results from the incorporation of a modified nucleoside triphosphate during an amplification reaction and therefore becoming part of the nucleic acid polymer.

Nucleoside -- is a base-sugar combination or a nucleotide lacking a phosphate moiety.

Nucleotide polymerase -- a catalyst, usually an enzyme, for forming an extension of a polynucleotide along a DNA or RNA template where the extension is complementary thereto. The nucleotide polymerase is a template dependent polynucleotide polymerase and utilizes nucleoside triphosphates as building blocks for extending the 3'-end of a polynucleotide to provide a sequence complementary with the polynucleotide template. Usually, the catalysts are enzymes, such as DNA polymerases, for example, prokaryotic DNA polymerase (I, II, or III), T4 DNA polymerase, T7 DNA polymerase, Klenow fragment, reverse transcriptase, Vent DNA polymerase, Pfu DNA polymerase, Taq DNA polymerase, and the like, derived from any source such as cells, bacteria, such as E. coli, plants, animals, virus, thermophilic bacteria, and so forth. RNA polymerases include T7 RNA polymerase and Q-beta-replicase.

Wholly or partially sequentially -- when the sample and various agents utilized in the present invention are combined other than concomitantly (simultaneously), one or more may be combined with one or more of the remaining agents to form a subcombination. Subcombination and remaining agents can then be combined and can be subjected to the present method.

Hybridization (hybridizing) and binding -- in the context of nucleotide sequences these terms are used interchangeably herein. The ability of two nucleotide sequences to hybridize with each other is based on the degree of complementarity of the two nucleotide sequences, which in turn is based on the fraction of matched complementary nucleotide pairs. The more nucleotides in a given sequence that are complementary to another sequence, the more stringent the conditions can be for hybridization and the more specific will be the binding of

the two sequences. Increased stringency is achieved by elevating the temperature, increasing the ratio of cosolvents, lowering the salt concentration, and the like.

Homologous or substantially identical polynucleotides-- In general, two polynucleotide sequences that are identical or can each hybridize to the same polynucleotide sequence are homologous. The two sequences are homologous or substantially identical where the sequences each have at least 90%, preferably 100%, of the same or analogous base sequence where thymine (T) and uracil (U) are considered the same. Thus, the ribonucleotides A, U, C and G are taken as analogous to the deoxynucleotides dA, dT, dC, and dG, respectively. Homologous sequences can comprise DNA, RNA or modified polynucleotides and may be homoduplexes, for example, RNA:RNA and DNA:DNA or heteroduplexes, for example, RNA:DNA.

Complementary -- two sequences are complementary when the sequence of one can bind to the sequence of the other in an anti-parallel sense wherein the 3'-end of each sequence binds to the 5'-end of the other sequence and each A, T (U), G, and C of one sequence is then aligned with a T (U), A, C, and G, respectively, of the other sequence.

Non-contiguous -- two sequences within a single polynucleotide sequence are non-contiguous when the 5' end of one sequence is joined to the 3' end of the other sequence by a chain of one or more nucleotides.

Contiguous -- two sequences within a single polynucleotide strand are contiguous when the 5' end of one sequence is joined directly to the 3' end of the other sequence without an intervening chain of nucleotides.

Copy of a sequence -- a sequence that is a direct identical copy of a single stranded polynucleotide sequence as differentiated from a sequence that is complementary to the sequence of such single stranded polynucleotide.

Means for extending a primer -- a nucleotide polymerase or a single stranded template polynucleotide having a sequence other than at its 3'-end that can hybridize to at least the 3'-end of the primer or both. Means for extending a primer also includes nucleoside triphosphates or analogs thereof capable of acting as substrates for the enzyme and other materials and conditions required for enzyme

activity such as a divalent metal ion (usually magnesium), pH, ionic strength, organic solvent (such as formamide), and the like.

Member of a specific binding pair ("sbp member")--one of two different molecules, having an area on the surface or in a cavity which specifically binds to and is thereby defined as complementary with a particular spatial and polar organization of the other molecule. The members of the specific binding pair are referred to as ligand and receptor (antiligand). These may be members of an immunological pair such as antigen-antibody, or may be operator-repressor, nuclease-nucleotide, biotin-avidin, hormones-hormone receptors, nucleic acid duplexes, IgG-protein A, DNA-DNA, DNA-RNA, and the like.

Ligand -- any compound for which a receptor naturally exists or can be prepared.

Receptor ("antiligand") -- any compound or composition capable of recognizing a particular spatial and polar organization of a molecule, e.g., epitopic or determinant site. Illustrative receptors include naturally occurring receptors, e.g., thyroxine binding globulin, antibodies, enzymes, Fab fragments, lectins, nucleic acids, repressors, protection enzymes, protein A, complement component C1q, DNA binding proteins or ligands and the like.

Small organic molecule -- a compound of molecular weight less than 1500, preferably 100 to 1000, more preferably 300 to 600 such as biotin, fluorescein, rhodamine and other dyes, tetracycline and other protein binding molecules, and haptens, etc. The small organic molecule can provide a means for attachment of a nucleotide sequence to a label or to a support.

Support or surface -- a porous or non-porous water insoluble material. The support can be hydrophilic or capable of being rendered hydrophilic and includes inorganic powders such as silica, magnesium sulfate, and alumina; natural polymeric materials, particularly cellulosic materials and materials derived from cellulose, such as fiber containing papers, e.g., filter paper, chromatographic paper, etc.; synthetic or modified naturally occurring polymers, such as nitrocellulose, cellulose acetate, poly (vinyl chloride), polyacrylamide, cross linked dextran, agarose, polyacrylate, polyethylene, polypropylene, poly(4-methylbutene),

polystyrene, polymethacrylate, poly(ethylene terephthalate), nylon, poly(vinyl butyrate), etc.; either used by themselves or in conjunction with other materials; glass available as Bioglass, ceramics, metals, and the like. Natural or synthetic assemblies such as liposomes, phospholipid vesicles, and cells can also be employed.

Binding of sbp members to a support or surface may be accomplished by well-known techniques, commonly available in the literature. See, for example, "Immobilized Enzymes," Ichiro Chibata, Halsted Press, New York (1978) and Cuatrecasas, J. Biol. Chem., 245:3059 (1970). The surface can have any one of a number of shapes, such as strip, rod, particle, including bead, and the like.

Label -- a member of a signal producing system. Usually the label is part of an oligonucleotide probe either being conjugated thereto or otherwise bound thereto or associated therewith and is capable of being detected directly or indirectly. Labels include reporter molecules that can be detected directly by virtue of generating a signal, specific binding pair members that may be detected indirectly by subsequent binding to a cognate that contains a reporter molecule, a specific polynucleotide sequence or recognition sequence that can act as a ligand such as for a repressor protein, wherein in the latter two instances the oligonucleotide primer or repressor protein will have, or be capable of having, a reporter molecule.

The label is a member of a signal producing system and can generate a detectable signal either alone or together with other members of the signal producing system. As mentioned above, a reporter molecule can be bound directly to a nucleotide sequence or can become bound thereto by being bound to an sbp member complementary to an sbp member that is bound to a nucleotide sequence.

Signal Producing System -- the signal producing system may have one or more components, at least one component being a label. The signal producing system generates a signal that relates to the presence or amount of target polynucleotide in a sample. The signal producing system includes all of the reagents required to produce a measurable signal. The labels and other reagents of the signal producing system must be stable at the temperatures used in an

amplification of a target polynucleotide. Detection of the signal will depend upon the nature of the signal producing system utilized.

When it is desirable to detect more than one product, separate signal producing systems are required that at least must produce measurably different signals and differ from each other in at least one of the members of each system. Preferably, the signal producing systems are chosen such that the binding of the second and third oligonucleotide probes to their respective duplexes to form termolecular complexes produces distinctly different signals. For example, in one approach the second oligonucleotide probe and the third oligonucleotide probe each comprise a member of a different signal producing system and the signals are measured from the ternary complexes and a ratio of signals is determined. The first oligonucleotide probe may comprise a member of both signal producing systems. In another approach the first oligonucleotide probe in combination with each of the second oligonucleotide probe and the third oligonucleotide probe comprise members of different signal producing systems and the signals measured from the ternary complexes are used to determine a ratio of signals. In another approach the first oligonucleotide probe comprises a first member of a each of two signal producing systems and the second oligonucleotide probe and the third oligonucleotide probe each respectively comprise a second member of each of the signal producing systems. Preferably, when the first member is brought into close proximity with the second members of the signal producing system, a signal is produced.

A number of signal producing systems in accordance with the above may be employed. The following discussion is by way of illustration and not limitation. In one such system the first member is a catalyst such as an enzyme and the second members are catalysts such as enzymes that are different from the first enzyme and from each other and the products of the reaction of the enzyme comprising the first member are the substrates for the other of the enzymes. By employing different second enzymes signals are produced that can be differentiated and used to determine a ratio of signals that is related to concentration of the target polynucleotide.

A list of enzymes is found in U.S. Patent No. 4,299,916 at column 30 to column 33. As mentioned above, of particular interest in the subject invention is the use of coupled catalysts, usually two or more enzymes, where the product of one enzyme serves as the substrate of the other enzyme. One of the enzymes is used as the label in the first oligonucleotide probe. Different second enzymes are used in the second and third oligonucleotide probes. Normally, either one or more substrates for the first enzyme or different substrates that can be transformed to different products by each of the second enzymes will be used. The enzymatic reaction may involve modifying the substrate to yield a product that is the primary substrate of another enzyme or produce a product that is a member of a pair of enzyme substrates whose reaction with each other is catalyzed by the second enzyme. The first situation may be illustrated by glucose-6-phosphate being catalytically hydrolyzed by alkaline phosphatase to glucose, wherein glucose is a substrate for glucose oxidase. The second situation may be illustrated by glucose being oxidized by glucose oxidase to provide hydrogen peroxide which would enzymatically react with the signal generator precursor to produce a signal generator.

When one or more substrates are used for the first enzyme, one or more products must be formed that are converted to different products and can yield different detectable signals by each of the second enzymes. One approach is to use two substrates that are converted by the first enzyme to different products. For example, a mixture of p-propylphenyl phosphate and a phosphate ester of o-nitrophenyl-β-galactoside can be used as the substrates for the first enzyme, alkaline phosphatase. The products, p-propylphenyl and o-nitrophenyl-β-galactoside, then serve as substrates for horseradish peroxidase and β-galactosidase, respectively. The former provides a fluorescent signal and the later, a light absorption signal. Alternatively a single substrate for the first enzyme could be used. Thus p-nitrophenyl 6-glucosyl diphosphate could be used with a phosphodiesterase as the first enzyme. The products, glucose-6-phosphate and p-nitrophenyl phosphate are chromogenic substrates for G6PDH and alkaline

phosphatase, respectively, which can therefore serve as the second enzymes. The products absorb at different wavelengths.

When different substrates are used for the second enzymes, different products must be produced that will serve as substrates for the first enzyme. For example, 1-(β-galactosyloxy)-3-indole and 1-(β-glucosyloxy)-4-propylbenzene could be used as substrates for the second enzymes, β-galactosidase and β-glucosidase, respectively. Each of the corresponding products, 3-hydroxyindole and p-propylphenol will then serve as substrates for the first enzyme, horseradish peroxidase and will produce respectively a fluorescent and a light absorption signal.

Coupled catalysts can also include an enzyme with a non-enzymatic catalyst. The enzyme can produce a reactant that undergoes a reaction catalyzed by the non-enzymatic catalyst or the non-enzymatic catalyst may produce a substrate (includes coenzymes) for the enzyme. For example, Medola blue can catalyze the conversion of NAD and hydroquinones to NADH that reacts with FMN oxidoreductase and bacterial luciferase in the presence of long chain aldehydes to produce light. Examples of particular catalytic systems that may be utilized in the present invention are found in U.S. Patent No. 4,299,916 at column 33, line 34, to column 38, line 32, the disclosure of which is incorporated herein by reference. For enzyme labels, additional members of the signal producing system include enzyme substrates and so forth. The product of the enzyme reaction is preferably a luminescent product or a fluorescent or non-fluorescent dye, any of which can be detected spectrophotometrically, or a product that can be detected by other spectrometric or electrometric means.

In another approach the first member of the signal producing system is an energy donor or energy acceptor and the second members are fluorescent compounds that emit at different wavelengths or with different decay rates. Fluorescers of interest generally emit light at a wavelength above 350 nm, usually above 400 nm and preferably above 450 nm. Desirably, the fluorescers have a high quantum efficiency, and are chemically stable under the conditions of their conjugation and use. Normally, the energy donors absorb maximally at shorter wavelengths and the energy acceptors at longer wavelengths than the absorption

maxima of the fluorescers. Further, if the decay rates are used to differentiate the signals from the two fluorescers, the energy donors have shorter lifetimes than at least one of the fluorescers. The term fluorescer is intended to include substances that emit light upon activation and include fluorescent and phosphorescent substances, scintillators and chemiluminescent substances. In this approach the medium is irradiated with light and the different fluorescence signals are determined. As will be appreciated, when the energy donor or energy acceptor is brought into close proximity to the fluorescent molecule by the formation of a termolecular complex, the fluorescence is correspondingly increased or decreased because of the transfer of energy to or from the fluorescer.

Alternatively, the first member of the signal producing system is a fluorescer and the second members are energy donors. Although the fluorescence spectrum is the same regardless of which member donates energy, the lifetime of the fluorescence reflects the lifetime of the excited state of the donor. Signals from the two donors can therefore be determined by measuring he fluorescence decay rates.

Fluorescers of interest fall into a variety of categories having certain primary functionalities. These primary functionalities include 1- and 2-aminonaphthalene, p,p-diaminostilbenes, pyrenes, quaternary phenanthridine salts, 9-aminoacridines, imines, anthracenes, oxacarboxyamine, merocyanine, 3-aminoequilenin, perylene, bis-benzoxazole, bis-p-oxazolyl benzene, 1,2-benzophenazine, retinal, bis-3-aminopyridinium salts, hellebrigenin, tetracycline, sterophenol, benzimidazolylphenylamine, 2-oxo-3-chromen, indole, xanthene, 7-hydroxycoumarin, 4,5-benzimidazoles, phenoxazine, salicylate, strophanthidin, porphyrins, triarylmethanes, flavin and rare earth chelates oxides and salts. Exemplary fluorescers are enumerated in U.S. Patent No. 4,318,707 at columns 7 and 8, the disclosure of which is incorporated herein by reference.

A diverse number of energy donors and acceptors may be employed. The energy donor or acceptor must be able to transfer energy to or from the fluorescer when brought into proximity with the fluorescer by virtue of the binding of the probes. Energy donors and acceptors are chromophores having substantial absorption

higher than 310 nm, normally higher than 350 nm, and preferably higher than about 400 nm. Generally, they are fluorescent compounds but they may have weak or no fluorescence and still be useful. For example, one group includes xanthene dyes, which include the fluoresceins derived from 3,6-dihydroxy-o-phenyl-xanthhydrol and rhodamines, derived form 3,6-diamino-9-phenylxanthhydrol. Another group includes chelates of fluorescent lanthanides such as Eu and Sm. Another group includes the naphthylamines such as, e.g., 1-anilino-8-naphthalene sulfonate, 1-dimethylaminonaphthyl-5-sulfonate and the like. Another group includes natural proteinaceous pigments such as the phycobiliproteins and green fluorescent proteins. Other examples of energy donors and acceptors that may be employed are those fluorescers of interest mentioned above wherein one fluorescer can absorb the energy of another fluorescer.

Energy acceptors that are non-fluorescent can include any of a wide variety of azo dyes, cyanine dyes, 4,5-dimethoxyfluorescein, formazans, indophenols and the like.

Another example of quenchers is energy absorbent or quenching particles. Examples of such particles are carbon particles, such as charcoal, lampblack, graphite, colloidal carbon and the like. Besides carbon particles metal sols may also find use, particularly of the noble metals, gold, silver, and platinum. Other metal derived particles may include metal sulfides, such as lead, silver or copper sulfides or metal oxides, such as iron or copper oxide.

As mentioned above, Heller (U.S. Patent No. 5,565,322) discloses donor and acceptor chromophores at column 9, line 37, to column 14, line 7, the disclosure of which is incorporated herein by reference. A further discussion of fluorescers and quenchers may also be found in U.S. Patent Nos. 4,261,968, 4,174,384, 4,199,983 and 3,996,345, the relevant disclosures of which are incorporated herein by reference.

In another approach the first member of the signal producing system is a sensitizer and the second members are chemiluminescent compounds that emit at different wavelengths or with different decay rates. Alternatively, the first member is a chemiluminescent compound and the second members are sensitizers that can be

independently excited by different wavelengths of light. Examples of chemiluminescent compounds and sensitizers are set forth in U.S. Serial No. 07/923,069 filed July 31, 1992, the disclosure of which is incorporated herein by reference. Particularly preferred are photosensitizers and photoactivatable chemiluminescent compounds such as described in U.S. Patent No. 5,340,716 at column 19, line 30, to column 20, line 45, and column 22, line 58, to column 30, line 10, the disclosure of which is incorporated herein by reference. The sensitizers are those compounds that generate singlet oxygen usually by excitation with light. The sensitizer can be photoactivatable (e.g., dyes and aromatic compounds) or chemi-activated (e.g., enzymes and metal salts). Typical photosensitizers include acetone, benzophenone, 9-thioxanthone, eosin, 9,10-dibromoanthracene, methylene blue, metallo-porphyrins, such as hematoporphyrin, phthalocyanines, chlorophylls, rose bengal, buckminsterfullerene, etc., and derivatives thereof. Photoactivatable chemiluminescent compounds are substances that undergo a chemical reaction upon direct of sensitized excitation by light of upon reaction with singlet oxygen to form a metastable reaction product that is capable of decomposition with the simultaneous or subsequent emission of light, usually with the wavelength range of 250 to 1200 nm. Preferably, these compounds react with singlet oxygen to form dioxetanes or dioxetanones. The latter are usually electron rich olefins. Exemplary of such olefins is enol ethers, enamines, 9-alkylidene-N-alkylacridans, arylvinylethers, dioxenes, arylimidazoles, 9-alkylidene-xanthanes and lucigenin. Other compounds include luminol and other phthalhydrazides and chemiluminescent compounds that are protected from undergoing a reaction such as firefly luciferin, aquaphorin, luminol, etc.

Other components of the signal producing system may include substrates, enhancers, activators, chemiluminescent compounds, cofactors, inhibitors, scavengers, metal ions, specific binding substances required for binding of signal generating substances, and the like. Other components of the signal producing system may be coenzymes, substances that react with enzymic products, other enzymes and catalysts, and the like.

Termolecular complex -- a ternary complex formed in accordance with the present methods upon the binding of the first oligonucleotide probe to the target polynucleotide and the binding of the second oligonucleotide probe to the duplex formed thereby. Such complex is termolecular in that it involves three molecules, namely, the two oligonucleotide probes and the target polynucleotide. Similarly, the first oligonucleotide probe binds to the reference polynucleotide and the third oligonucleotide probe binds to the duplex formed thereby. As will be appreciated, such a termolecular complex is formed for each reference polynucleotide used in the present invention.

Ancillary materials -- Various ancillary materials will frequently be employed in the methods and assays carried out in accordance with the present invention. For example, buffers will normally be present in the assay medium, as well as stabilizers for the assay medium and the assay components. Frequently, in addition to these additives, proteins may be included, such as albumins, organic solvents such as formamide, quaternary ammonium salts, polycations such as dextran sulfate, surfactants, particularly non-ionic surfactants, binding enhancers, e.g., polyalkylene glycols, or the like.

A mentioned above, in one broad aspect the present invention is directed to a method for detecting a single stranded target polynucleotide. A ternary complex is formed comprising the target polynucleotide and first and second oligonucleotide probes, which, respectively, have different first sequences that are complementary to the target polynucleotide and different second sequences that are complementary to each other. The complex is formed under conditions wherein the first and second oligonucleotide probes do not substantially bind to each other in the absence of the target polynucleotide. The association of the first and second oligonucleotide probes is then detected.

This aspect of the present invention is depicted in Fig. 1. Target polynucleotide T comprises sequences T1 and T2, which is 5' of T1 and contiguous therewith in the embodiment depicted. A first oligonucleotide probe PT comprises sequences PT3 and PT1, which is 5' of PT3 and contiguous therewith. PT1 of the first oligonucleotide probe is capable of hybridizing to T1 of the target

polynucleotide. PT has label L2 at its 3' end. The target polynucleotide and the first oligonucleotide probe form a duplex TPT by virtue of the hybridization of PT 1 with T1. A second oligonucleotide probe P comprises sequences P2 and P3, which is 5' of P2. P2 is capable of hybridizing to T2 of the target polynucleotide and P3 is capable of hybridizing to PT3 of the first oligonucleotide probe. The second oligonucleotide probe P binds to duplex TPT to form termolecular complex TPTP by virtue of the hybridization of PT3 with P3 and P2 with T2. P also has a label L1 at its 5' end. The formation of TPTP is detected and is indicative of the presence of the single stranded target polynucleotide. In the embodiment depicted in Fig. 1, the association of labels L1 and L2 in the termolecular complex is determined as a means of detecting the formation of TPTP.

As mentioned above one aspect of the present invention provides for detection of a target polynucleotide and its products produced in a nucleic acid amplification reaction. The present invention has particular application to amplification reactions conducted at isothermal temperature. When an amplification is employed, all of the necessary reagents for amplification and detection can be included in the reaction mixture prior to amplification and it is not necessary to open the reaction vessel after amplification and prior to detection. Thus, contamination is avoided. At the very least, complexes containing labels and any remaining members of the signal producing system can be added after amplification but without a separation step prior to detection.

The combination of reagents in a single reaction container, if desired, is subjected to conditions for amplifying the target polynucleotide sequence to form copies or complements thereof. For quantitative determination of a target polynucleotide, the reagents comprise at least first, second and third polynucleotide probes and one or more reference polynucleotides. The target polynucleotide has sequences T1 and T2, one of which is independently about 5 to 20 nucleotides in length and the other of which is 8 to 50 or more nucleotides in length. In general, for T1 and T2, and the other sequences referred to hereinbelow that are found within the respective reference polynucleotide and the first, second and third oligonucleotide probes, the lengths are such that, under the conditions of the

reactions involved, respective hybridizable pairs thereof do not form stable duplexes. T1 generally lies contiguous with T2 or within a relatively few nucleotides, preferably, less than 10 nucleotides, more preferably, 1 to 5 nucleotides, of T2.

A reference polynucleotide has a portion R2 that contains a nucleotide sequence that is identical to a region T2 of the target polynucleotide and a portion R1 located at approximately the same position relative to R2 as T1 is located relative to T2. The length of R1 and R2 are usually within 2 to 3 nucleotides of the lengths of T1 and T2, respectively. R1 contains a nucleotide sequence that is different than T1 and preferably not contained in the target polynucleotide of in any other reference polynucleotide that may be present. R1 and R2 have similar characteristics to T1 and T2, respectively, of the target polynucleotide. R1 generally lies contiguous with R2 or within a relatively few unhybridized nucleotides, preferably, less than 10 hybridized nucleotides, more preferably, 1 to 5 unhybridized nucleotides, of R2.

The first oligonucleotide probe is a universal probe in that it has a sequence that binds to a sequence within the target polynucleotide and sequences within all of the reference polynucleotides used in the present method. The first oligonucleotide probe comprises a sequence of nucleotides P2 that is hybridizable with, preferably, complementary to and the same length as, R2 and T2. The first oligonucleotide probe also has a sequence P3 that is not found in the target polynucleotide or in any of the reference polynucleotides that may be employed. P3 is about 5 to 20 nucleotides in length, preferably, about 8 to 16 in length. Sequence P3 is contiguous with P2 or is located within a few unhybridized nucleotides of P2, preferably, within less than 10 unhybridized nucleotides, more preferably, within 1 to 5 unhybridized nucleotides, of P2. Alternatively, there may be a longer sequence of nucleotides within the first oligonucleotide probe between P2 and P3 that forms a hairpin loop with the nucleotides comprising P2 and P3 immediately before and after the loop, respectively.

The second oligonucleotide probe PT has a sequence PT1 that is hybridizable with, preferably, complementary to and the same length as, T1 and a sequence PT3 that is hybridizable with, preferably complementary to and the same

length as, P3. The degree of hybridization of PT3 to P3 should be sufficient to form a stable termolecular complex. The level of hybridization does not need to be as stringent as with the hybridization of PT1 with T1, but in a preferred embodiment PT3 is complementary to P3. PT1 and PT3 are each independently about 5 to 20 nucleotides in length, preferably, about 8 to about 16 nucleotides in length. Sequence PT3 is contiguous with PT1 or is located within a few unhybridized nucleotides of PT1, preferably, within less than 10 unhybridized nucleotides, more preferably, within 1 to 5 unhybridized nucleotides, of PT1. Alternatively, there may be a longer sequence of nucleotides within the second oligonucleotide probe between PT1 and PT3 that forms a hairpin loop with the nucleotides comprising PT1 and PT3 immediately before and after the loop, respectively.

The third oligonucleotide probe PR has a sequence of nucleotides PR1 that is hybridizable with, preferably, complementary to and the same length as, reference sequence R1 and a sequence PR3 that is hybridizable with, preferably, complementary to, P3 of the first oligonucleotide probe and thus similar to or identical to PT3. The degree of hybridization of PR3 to P3 should be sufficient to form a stable termolecular complex. The level of hybridization does not need to be as stringent as with the hybridization of PR1 with R1, but in a preferred embodiment PR3 is complementary to P3. PR1 and PR3 are each independently about 5 to 20 nucleotides in length, preferably, about 8 to about 16 nucleotides in length. Sequence PR3 is contiguous with PR1 or is located within a few unhybridized nucleotides of PR1, preferably, within less than 10 unhybridized nucleotides, more preferably, within 1 to 5 unhybridized nucleotides, of PR1. Alternatively, there may be a longer sequence of nucleotides within the third oligonucleotide probe between PR3 and PR1 that forms a hairpin loop with the nucleotides comprising PR1 and PR3 immediately before and after the loop, respectively.

Furthermore, if T1 and R1 are 3' of T2 and R2, respectively, then PR3 must be 3' of PR1 and PT3 must be 3' of PT1. In addition, P2 must be 3' of P3. The situation is reversed where T1 and R1 are 5' of T2 and R2.

It should be noted that the length of T1, T2, P1, P2, P3, PT1, PT2, PT3, PR1, PR2 and PR3 may be greater or less than that indicated above. The length

depends on a number of factors such as the possibility of strand invasion, whether the nucleotides are natural or modified, the temperature, the pH, the salt concentration of the medium, the nature of the bridge, and so forth.

One of the oligonucleotide probes in each termolecular complex is incapable of independently forming a stable duplex with the other probe or with the reference or target polynucleotides under the reaction conditions. This result is achieved primarily by selecting the length of each of the sequences or using modified nucleotides in one of the oligonucleotide probes such that each is too short or binds too weakly to form a stable duplex under the conditions employed. Thus, the probes are only able to bind to each other as ternary complexes with the reference and target polynucleotides and excess target and reference polynucleotides cannot interfere with the amount of the ternary complexes that are formed.

An example of the above is depicted in Fig. 2 by way of illustration and not limitation. Target polynucleotide T comprises sequences T1 and T2, which is 5' of T1 and contiguous therewith in the embodiment depicted. Reference polynucleotide R comprises sequences R1 and R2, which is 5' of R1 and contiguous therewith. The target and reference polynucleotides are combined with first oligonucleotide probe U comprising sequences P2 and P3, which is 5' of P2 as shown and also contiguous with P2. The first oligonucleotide probe also comprises a first member of a signal producing system designated L1. The combination produces duplex TU wherein P2 hybridizes with T2. Duplex RU is also produced wherein P2 hybridizes with R2. Also in the combination are second (PTU) and third (PRU) oligonucleotide probes. The second oligonucleotide probe PTU comprises sequences PT3 and PT1 wherein PT1 is 5' of PT3 and contiguous therewith. PTU also comprises a second member of a signal producing system designated L2. PT1 of the second oligonucleotide probe hybridizes to T1 of the duplex to give termolecular complex TUPTU.

The combination also includes a third oligonucleotide probe PRU comprising sequences PR3 and PR1. PR3 is essentially identical to the sequence PT3 of the second oligonucleotide probe. PR3 hybridizes to P3 and PR1 hybridizes to R1 of the duplex RU, which includes the reference polynucleotide. The third

oligonucleotide probe also comprises a second member L2' of a signal producing system, which is different from the second member of the signal producing system for the second oligonucleotide probe. As explained above, the signal producing systems, of which the second member is a part, differ in signal production so that the signals can be differentiated and a ratio of the signals can be made to determine the concentration of the target polynucleotide. The third oligonucleotide hybridizes to duplex RU by virtue of the hybridization of PR1 with R1 of the reference oligonucleotide and PR3 with P3 of the first oligonucleotide probe. In this way termolecular complex RUPRU is formed. The signals produced by the signal producing systems are detected and a signal ratio is determined and related to the amount of the target polynucleotide.

Fig. 3 depicts an embodiment wherein the respective sequences in the target polynucleotide, the reference polynucleotide and the first, second and third oligonucleotides are not contiguous. As mentioned above, when the respective sequences are not contiguous, the sequences are separated by no greater than 10 unhybridized nucleotides.

Fig. 4 depicts an embodiment wherein the sequence of nucleotides between P2 and P3 of the first oligonucleotide probe includes a hairpin loop. Accordingly, there are more than 10 nucleotides between P2 and P3. However, there are no greater than 10 unhybridized nucleotides between P2 and P3 because the additional nucleotides hybridize to one another, i.e., self-hybridize, to form a hairpin loop.

Fig. 5 depicts an embodiment of the present invention wherein two reference polynucleotides R^1 and R^2 are utilized. R^1 is similar to R in Fig. 2 and comprises sequences $R^1$1 and $R^1$2, which is 5' of $R^1$1 and contiguous therewith. A second reference polynucleotide R^2 is used and comprises sequences $R^2$1 and $R^2$2, which is 5' of $R^2$1 and contiguous therewith. $R^1$2 and $R^2$2 are essentially the same as T2 and $R^1$1 and $R^2$1 are different from one another and from sequence T1 of the target. The target and reference polynucleotides are combined with first oligonucleotide probe U comprising sequences P2 and P3, which is 5' of P2 as shown and also contiguous with P2. The first oligonucleotide probe also comprises a first member of a signal producing system designated L1. The combination produces duplex TU

wherein P2 hybridizes with T2. Duplexes R^1U and R^2U are also produced wherein P2 hybridizes with $R^1$2 and with $R^2$2. Also in the combination are second oligonucleotide probe (PTU), third oligonucleotide probe (PR^1U) and fourth oligonucleotide probe (PR^2U). The second oligonucleotide probe PTU comprises sequences PT3 and PT1 wherein PT1 is 5' of PT3 and contiguous therewith. PTU also comprises a second member of a signal producing system designated L2. PT1 of the second oligonucleotide probe hybridizes to T1 and PT3 hybridizes to P3 of the duplex TU to give termolecular complex TUPTU.

The combination also includes a third oligonucleotide probe PR^1U comprising sequences $PR^1$3 and $PR^1$1. $PR^1$3 is at least capable of hybridizing with P3 and is normally essentially identical to PT3 of the second oligonucleotide probe. $PR^1$1 is capable of hybridizing to $R^1$1 and $PR^1$3 is capable of hybridizing with P3 of the duplex R^1U. The third oligonucleotide probe also comprises a second member L2' of a signal producing system, which is different from the second member of the signal producing system for the second oligonucleotide probe.

The combination also includes a fourth oligonucleotide probe PR^2U comprising sequences $PR^2$3 and $PR^2$1. $PR^2$3 is at least capable of hybridizing with P3 and is normally essentially identical to PT3 of the second oligonucleotide probe and $PR^1$3 of the third oligonucleotide probe. $PR^2$1 is capable of hybridizing to $R^2$1 and $PR^2$3 is capable of hybridizing with P3 of the duplex R^2U. The fourth oligonucleotide probe also comprises a second member L2" of a signal producing system, which is different from the second member of the signal producing system L2 for the second oligonucleotide probe and the second member of the signal producing system L2' for the third oligonucleotide probe.

As explained above, the signal producing systems, of which the second member is a part, differ in signal production so that the signals can be differentiated and a ratio of the signals can be made to determine the concentration of the target polynucleotide. The third oligonucleotide probe hybridizes to duplex R^1U by virtue of the hybridization of $PR^1$1 with $R^1$1 of the reference oligonucleotide and $PR^1$3 with P3 of the first oligonucleotide probe. In this way termolecular complex $RUPR^1U$ is formed. The fourth oligonucleotide probe hybridizes to duplex R^2U by virtue of the

hybridization of PR^21 with R^21 of the reference oligonucleotide and PR^23 with P3 of the first oligonucleotide probe. In this way termolecular complex $RUPR^2U$ is formed. The signals produced by the signal producing systems are detected and signal ratios are determined and related to the amount of the target polynucleotide.

When the oligonucleotide probes are present in the amplification mixture, preferably, they are blocked at the 3'-end to avoid any potential interference with and during amplification. To this end, the 3'-end can be blocked by a group that cannot undergo chain extension, such as, for example, a 3'-phosphate, a 3'-terminal dideoxy, an abasic ribophosphate, a polymer or surface, or other means for inhibiting 3' end reactions. Alternatively, a polynucleotide that does not hybridize to the target or the reference polynucleotide is attached to the 3'-end. Such an end group can be introduced at the 3' end during solid phase synthesis or a group can be introduced that can subsequently be modified. For example, in order to introduce dextran at the 3'-end, a ribonucleotide can be introduced at the 3'-end and then oxidized with periodate followed by reductive amination of the resulting dialdehyde with borohydride and aminodextran. The details for carrying out the above modifications are well known in the art and will not be repeated here.

In carrying out an amplification as part of the present method, an aqueous medium is employed. Other polar cosolvents may also be employed, usually oxygenated organic solvents of from 1-6, more usually from 1-4, carbon atoms, including alcohols, ethers and the like. Usually these cosolvents, if used, are present in less than about 70 weight percent, more usually in less than about 30 weight percent.

The pH for the medium is usually in the range of about 4.5 to 9.5, more usually in the range of about 5.5 to 8.5, and preferably in the range of about 6 to 8. Various buffers may be used to achieve the desired pH and maintain the pH during the determination. Illustrative buffers include borate, phosphate, carbonate, Tris, barbital and the like. The particular buffer employed is not critical to this invention but in individual methods one buffer may be preferred over another. In general for amplification, the pH and temperature are chosen based on the particular method of amplification employed. For example, for amplification involving temperature cycling

and primer extension such as in PCR or single primer amplification, the pH and the temperature are selected so as to cause, either simultaneously or sequentially, dissociation of any internally hybridized sequences, hybridization of the oligonucleotide primer with the target polynucleotide sequence, extension of the primer, and dissociation of the extended primer. This usually involves cycling the reaction medium among two or more temperatures. In conducting PCR amplification of nucleic acids, the medium is cycled between two to three temperatures. The temperatures for PCR amplification generally range from about 50°C to 100°C, more usually, from about 60°C to 95°C. Relatively low temperatures of from about 50°C to 80°C are employed for the hybridization steps, while denaturation is carried out at a temperature of from about 80°C to 100°C and extension is carried out at a temperature of from about 70°C to 80°C, usually about 72°C to 74°C.

For amplification by NASBA and 3SR, the reaction is conducted at isothermal temperature, which is usually about 38 to 44°C, preferably about 41°C.

The amplification is conducted for a time sufficient to produce the desired number of complements or copies of the target polynucleotide. This, in turn, depends on the type of amplification reaction and the purpose for which the amplification is conducted, such as, for example, an assay for a polynucleotide analyte. Generally, the time period for conducting the entire method will be from about 10 to 200 minutes. As a matter of convenience, it will usually be desirable to minimize the time period. For amplification involving temperature cycling, the time is about 10 to 200 seconds per cycle and any number of cycles can be used from 1 to as high as 200 or more, usually 5 to 80, frequently 10-60. The time period for amplification involving isothermal temperatures is usually about 10 to 40 minutes.

The concentration of the nucleotide polymerase is usually determined empirically. Preferably, a concentration is used that is sufficient such that further increase in the concentration does not decrease the time for the amplification by over 5-fold, preferably 2-fold. The primary limiting factor generally is the cost of the reagent.

The amount of the target polynucleotide to be amplified can be as low as one or two molecules in a sample but generally may vary from about 10 to 10^{10}, more usually from about 10^3 to 10^8 molecules in a sample preferably at least 10^{-21}M in the sample and may be 10^{-10} to 10^{-19}M, more usually 10^{-14} to 10^{-19}M. For amplification by primer extension, the amount of the oligonucleotide primer(s) will be at least as great as the number of copies desired and will usually be 10^{-13} to 10^{-8} moles per sample, where the sample is 1-1,000 μL. Usually, the primer(s) are present in at least 10^{-9} M, preferably 10^{-7} M, and more preferably at least about 10^{-6} M. Preferably, the concentration of the oligonucleotide primer(s) is substantially in excess over, preferably at least 100 times greater than, more preferably, at least 1000 times greater than, the concentration of the target polynucleotide sequence.

For amplifications involving primer extension, the concentration of the nucleoside triphosphates in the medium can vary widely; preferably, these reagents are present in an excess amount. The nucleoside triphosphates are usually present in 10^{-6} to 10^{-2}M, preferably 10^{-5} to 10^{-3}M.

The order of combining of the various reagents to form the combination may vary. When no amplification is used the target polynucleotide is combined with the oligonucleotide probes and optionally with a reference polynucleotide. Usually, the mixture is heated to denature the target when the target is not single stranded and the temperature is then adjusted to permit binding of the probes with the target. When amplification using a DNA polymerase is required, generally, the target polynucleotide is obtained from a sample containing the polynucleotide analyte that has been treated to obtain such target polynucleotide. When a reference polynucleotide is used, it is generally combined with the sample prior to treatment to extend the target polynucleotide. Generally, the target and the reference polynucleotides are then combined with a pre-prepared combination of nucleoside triphosphates and nucleotide polymerase. The oligonucleotide primer(s) and the oligonucleotide probes may be included in the prepared combination or may be added subsequently. However, simultaneous addition of all of the above, as well as other step-wise or sequential orders of addition, may be employed provided that all

of the reagents described above are combined prior to the start of the amplification and other reactions.

Generally, it is desirable to increase the number of complements and/or copies of the target polynucleotide by at least a factor of 10^2, preferably a factor of 10^4, more preferably 10^6 or more.

After appropriate amplification, the medium is next examined to determine the presence of the target polynucleotide. In accordance with the present invention the reference polynucleotide and the first and second oligonucleotide probes and, optionally, the third oligonucleotide probe, which are already present in the reaction medium, are used for the determination. To this end the reaction medium is subjected to conditions to allow for the binding of the first oligonucleotide probe to the target and reference polynucleotides, respectively, to form duplexes and for the binding of the second and third oligonucleotide probes to the respective duplexes to form termolecular complexes. Alternatively, the second and the third oligonucleotide probes are allowed to bind to the target and the reference polynucleotides, respectively, to form duplexes; and the first oligonucleotide probe is allowed to bind to the respective duplexes to form termolecular complexes. Usually, this is achieved by adjusting the temperature of the reaction medium to allow for the formation of the appropriate complexes. The temperature chosen is dependent on the structure of the oligonucleotide probes and the nucleotide sequence of the amplicon. In general, the temperature for this aspect of the present invention is below 75°C, preferably, 20°C to 65°C, usually, 30°C to 55°C. The structure of the probes and the amplicon affect the choice of temperature, which is determined empirically. In general, shorter sequences permit lower temperatures to be used for hybridization.

When the present invention is employed to determine the amount of a target polynucleotide in a medium, a predetermined amount of the reference polynucleotide is employed per a measured amount of a medium containing the target polynucleotide. In general, the amount of the reference polynucleotide is at least the minimum amount that will permit detection following amplification. Often, several reference polynucleotides are used, each at a level of about 10 to 100 fold

higher than the next. The reference polynucleotides may therefore be present in as little as about 10 copies up to 10^7 or more copies per sample.

As mentioned above, a particular advantage of the present invention is that excess target polynucleotide does not interfere with the amount of the ternary complex formed. This results from the fact that one of the oligonucleotide probes can only bind to the duplexes of the other oligonucleotide probe with the target and with the reference polynucleotide. Thus, none of the probes need be present in excess concentration compared to the concentration of the amplification products of the reference polynucleotide and the target polynucleotide. The ratio of the amount of this probe bound to the two duplexes reflects the ratio of the amounts of target and reference polynucleotide present prior to amplification. Nevertheless, the use of relatively high concentrations of the oligonucleotide probes assists in achieving hybridization of the probes with the respective duplexes of the target polynucleotide and the reference polynucleotide during the detection part of the present method and increases the dynamic range of detection. In general, the concentration of the first, second and third, and so forth, oligonucleotide probes is limited by their cost and by possible inhibiting effects on the amplification. Usually, the probe concentrations are 10^{-10} to 10^{-5} M, more frequently, 10^{-8} to 10^{-6} M.

Detection of the termolecular complex is accomplished by using a signal producing system as described above. The signals are then differentiated and a ratio is determined and related to the presence and amount of the target polynucleotide.

A particular example of the present method as applied to the detection of a DNA target polynucleotide in a sample suspected of containing the target polynucleotide is next described by way of illustration and not limitation. The amplification method chosen is PCR. Accordingly, the sample suspected of containing the target polynucleotide is combined in a reaction medium with all of the reagents for conducting a PCR amplification, namely, a DNA polymerase, nucleoside triphosphates and two oligonucleotide primers for providing extension products based on the DNA target polynucleotide template. These reagents are provided in amounts to achieve a concentration of the target polynucleotide, if present, in the reaction

medium after PCR amplification of at least about 10^{-12} molar, typically, about 10 to 100mM. Also included in the reaction medium are reference polynucleotides A and B that have similar sequences to the target polynucleotide except that 15 nucleotide sequences R_1^A and R_1^B replace a 15 nucleotide sequence T1 of the target polynucleotide. The predetermined amount of reference polynucleotide A added to the sample is 100 copies and the predetermined amount of reference polynucleotide B added to the sample is about 1000 copies. The reaction medium further contains a first, universal, oligonucleotide probe C, which has the photosensitizer, phthalocyanine, attached to a terminal nucleotide thereof at the 5' end. The medium also includes a second oligonucleotide probe D that has a sequence complementary to the sequence T1 in the target polynucleotide, a third oligonucleotide probe E that has a sequence complementary to the sequence R_1^A in reference polynucleotide A, and a fourth oligonucleotide probe F that is complementary to has the sequence R_1^B sequence in reference polynucleotide B. Universal probe C has a sequence P2 that is complementary to a 15 nucleotide sequence in each of the target and reference polynucleotides and a 10 nucleotide sequence that is complementary to a sequence common to each of the oligonucleotides D, E and F. Oligonucleotide probe D has a latex particle in which is dissolved a chemiluminescent dioxene attached to a terminal nucleotide at the 3' end thereof, oligonucleotide probe E has a latex particle in which is dissolved a chemiluminescent oxazine attached to a terminal nucleotide at the 3' end thereof and oligonucleotide F has a latex particle in which is dissolved a chemiluminescent thioxene attached to a terminal nucleotide at the 3' end thereof. The reaction medium is subjected to thermal cycling in a Perkin Elmer cycler as follows: 94°C (5 min.); 94°C (1 min.), 72°C (2 min.), for 25 cycles; 94°C (5 min.) 55°C (10 min.).

The detection and quantitation of the target polynucleotide is carried out at the end of the amplification or during the amplification. In the former situation, the amplification is carried out for the desired number of cycles and the reaction medium is brought to a temperature of 55°C for a period of 10 minutes. During this time the target polynucleotide, if present, hybridizes with C and with D to give a termolecular complex TCD. Likewise, reference polynucleotide A hybridizes with C and E to give

termolecular complex ACE, and reference polynucleotide B hybridizes with C and F to give termolecular complex BCF. The different signals from each of TCD, ACE and BCF are determined by repetitive irradiation for periods of one second followed by measurements of the rate of decrease of the resultant chemiluminescent signal and deconvolution of the rate and intensity of the signal based on the known differing chemiluminescent decay rates of each of the chemiluminescers to provide a ratio of the amounts of each of the chemiluminescers activated by the photosensitizer. These ratios relate directly to the ratio of the target polynucleotide to each of the reference polynucleotides in the sample.

Another particular example of the present method as applied to the detection of an RNA target polynucleotide in a sample suspected of containing the target polynucleotide is next described by way of illustration and not limitation. The amplification method chosen is NASBA. Accordingly, a measured amount of the sample suspected of containing the RNA target polynucleotide is combined with a predetermined amount of reference polynucleotides H and J. The predetermined amount of H is 100 copies and the predetermined amount of J is 1000 copies. The RNA is isolated from the sample and combined in a reaction medium with all of the reagents for conducting a NASBA amplification, namely, RNA polymerase, a DNA primer with a promoter sequence, a second DNA primer, T7 polymerase reverse transcriptase, RNAse-H, nucleotide triphosphates and deoxynucleoside triphosphates. These reagents are provided in amounts to achieve a concentration of the RNA target polynucleotide, if present, in the reaction medium after NASBA amplification of about 10^{-7} to 10^{-5} M. The reaction medium further contains a first oligonucleotide probe K, which is a universal probe and has the energy donor 7-dimethylaminocoumarin-3-carboxylic acid attached to a terminal nucleotide thereof at the 5' end. The medium also includes a second oligonucleotide probe K that has a sequence complementary to a sequence in the target polynucleotide, a third oligonucleotide probe L that has a sequence complementary to a sequence in reference polynucleotide M, and a fourth oligonucleotide probe N that has a sequence complementary to a sequence in reference polynucleotide H. Universal probe K has a sequence that is complementary to a sequence in the target

polynucleotide as well as oligonucleotides L, M and N. Oligonucleotide probe L has fluorescein attached to a terminal nucleotide at the 3' end thereof, oligonucleotide probe M has the fluorophore, phycoerythrin, attached to a terminal nucleotide at the 3' end thereof and oligonucleotide N has phycoerythrin conjugated to allophycocyanine attached to a terminal nucleotide at the 3' end thereof. The reaction medium is subjected to conditions for carrying out the NASBA amplification, namely, a temperature of 41°C for a time of 90 minutes.

As indicated above, the detection and quantitation of the target polynucleotide can be carried out at the end of the amplification or during the amplification. In the latter situation, the amplification is conducted and a determination of the signal is determined at a variety of points in time. This is achieved by irradiating the reaction medium at a wavelength of 415 nm and determining the ratios of the light emitted at three different wavelengths corresponding to the emission maxima of the three fluorophores fluorescein 520 nm, phycoerythrin 576 nm and phycoerythrin/allophycocyanine 660 nm. The target polynucleotide, if present, hybridizes with K and with L to give a termolecular complex TKL. Likewise, reference polynucleotide H hybridizes with K and M to give termolecular complex HKM, and reference polynucleotide J hybridizes with K and N to give termolecular complex JKN.

Briefly, detection using the induced luminescence assay as applied in the present invention involves an energy donor as part of one label and a fluorescent energy acceptor as part of the other label in a particular signal producing system. If the target polynucleotide is present the donor and the acceptor come into close proximity and the fluorescent acceptor is activated and emits light in relation to the amount of the complex formed.

As a matter of convenience, predetermined amounts of reagents employed in the present invention can be provided in a kit in packaged combination. A kit can comprise in packaged combination (a) reagents for conducting an amplification of the target polynucleotide, (b) a predetermined amount of a reference polynucleotide comprising a sequence R2 that is identical to T2 and a sequence R1 that is not present in the target polynucleotide, (c) a first oligonucleotide probe comprising a

first sequence P2 capable of hybridizing to R2 and T2 and a sequence P3 that is not capable of hybridizing to the target polynucleotide or the reference polynucleotide, a second oligonucleotide probe comprising a sequence PT1 capable of hybridizing to T1 and a sequence PT3 that is identical to PR3 and capable of hybridizing to P3, and a third oligonucleotide probe comprising a sequence PR1 that is capable of hybridizing to R1 and a sequence PR3 that is capable of hybridizing to P3. The probes contain members of a signal producing system as described above.

The kit can further include any additional members of a signal producing system and also various buffered media, some of which may contain one or more of the above reagents.

Another aspect of the present invention is a kit for use in an amplification and quantitative detection of a target RNA comprising sequences T1 and T2. The kit comprises in packaged combination: (a) a promoter, (b) an RNA polymerase, (c) a predetermined amount of a reference polynucleotide comprising a sequence R2 that is identical to T2 and a sequence R1 that is not present in the target RNA, (d) a first oligonucleotide probe comprising a first sequence P2 capable of hybridizing to R2 and T2 and a sequence P3 that is not capable of hybridizing to the target RNA or the reference polynucleotide, (e) a second oligonucleotide probe comprising a sequence PT1 capable of hybridizing to T1 and a sequence PT3 that is identical to PR3 and capable of hybridizing to P3, and (f) a third oligonucleotide probe comprising a sequence PR1 that is capable of hybridizing to R1 and a sequence PR3 that is capable of hybridizing to P3.

The relative amounts of the various reagents in the kits can be varied widely to provide for concentrations of the reagents necessary to achieve the objects of the present invention. Under appropriate circumstances one or more of the reagents in the kit can be provided as a dry powder, usually lyophilized, including excipients, which on dissolution will provide for a reagent solution having the appropriate concentrations for performing a method or assay in accordance with the present invention. Each reagent can be packaged in separate containers or some reagents can be combined in one container where cross-reactivity and shelf life permit. The

kits may also include a written description of a method in accordance with the present invention as described above.

EXAMPLES

The invention is demonstrated further by the following illustrative examples. Temperatures are in degrees centigrade (°C) and parts and percentages are by weight, unless otherwise indicated. Unless otherwise indicated, oligonucleotides used in the following examples were prepared by synthesis using an automated synthesizer and were purified by gel electrophoresis or HPLC.

The following abbreviations have the meanings set forth below:

Tris HCl - Tris(hydroxymethyl)aminomethane-HCl (a 10X solution) from BioWhittaker, Walkersville, MD.

DTT - dithiothreitol from Sigma Chemical Company, St. Louis, MO.

HPLC - high performance liquid chromatography.

DPP - 4,7-diphenylphenanthroline from Aldrich Chemical Company, Milwaukee WI.

BSA - bovine serum albumin from Sigma Chemical Company, St. Louis MO

ELISA - enzyme linked immunosorbent assay as described in "Enzyme-Immunoassay," Edward T. Maggio, CRC Press, Inc., Boca Raton, Florida (1980)

bp - base pairs

ddc - dideoxycytidine

g - grams

mmol - millimolar

DMF - dimethyl formamide

THF - tetrahydrofuran

LSIMS - fast ion bombardment mass spectroscopy

NMR - nuclear magnetic resonance spectroscopy

TMSCl - tetramethylsilylchloride

EDAC - 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride.

MES - 2-(N-morpholino)ethane sulfonic acid.

SPDP - N-succinimidyl 3-(2-pyridylthio)-propionate.

Sulfo-SMCC - 4-(N-maleimidomethyl)cyclohexane-1-carboxylate.

TCEP - tris-carboxyethyl phosphine.

PREPARATION OF REAGENTS

C-28 thioxene:

To a solution of 4-bromoaniline (30g, 174mmol) in dry DMF (200mL) was added 1-bromotetradecane (89.3mL, 366mmol) and N,N-diisopropylethylamine (62.2mL, 357mmol). The reaction solution was heated at 90°C for 16 hr under argon before being cooled to room temperature. To this reaction solution was again added 1-bromotetradecane (45mL, 184mmol) and N,N-diisopropylethylamine (31mL, 178mmol) and the reaction mixture was heated at 90°C for another 15 hr. After cooling, the reaction solution was concentrated in vacuo and the residue was diluted with CH_2Cl_2 (400mL). The CH_2Cl_2 solution was washed with 1N aqueous NaOH (2x), H_2O, and brine, was dried over Na_2SO_4 and was concentrated in vacuo to yield a dark brown oil (about 110g). Preparative column chromatography on silica gel by a Waters 500 Prep LC system eluting with hexane afforded a yellow oil that contained mainly the product (4-bromo-N,N-di-($C_{14}H_{29}$)-aniline) along with a minor component 1-bromotetradecane. The latter compound was removed from the mixture by vacuum distillation (bp 105-110°C, 0.6mm) to leave 50.2g (51%) of the product as a brown oil. To a mixture of magnesium turnings (9.60g, 395mmol) in dry THF (30mL) under argon was added dropwise a solution of the above substituted aniline product (44.7g, 79mmol) in THF (250mL). A few crystals of iodine were added to initiate the formation of the Grignard reagent. When the reaction mixture became warm and began to reflux, the addition rate was regulated to maintain a gentle reflux. After addition was complete, the mixture was heated at reflux for an additional hour. The cooled supernatant solution was transferred via cannula to an addition funnel and added dropwise (over 2.5 hr) to a solution of phenylglyoxal (11.7g, 87mmol) in THF (300mL) at -30°C under argon. The reaction mixture was gradually warmed to 0°C over 1 hr and stirred for another 30 min. The resulting mixture was poured into a mixture of ice water (800mL) and ethyl acetate (250mL).

The organic phase was separated and the aqueous phase was extracted with ethyl acetate (3x). The combined organic phases were washed with H_2O (2x), brine and was dried over $MgSO_4$. Evaporation of the solvent gave 48.8g of the crude product as a dark green oily liquid. Flash column chromatography of this liquid (gradient elution with hexane, 1.5:98.5, 3:97, 5:95 ethyl acetate:hexane) afforded 24.7g (50%) of the benzoin product (LSIMS ($C_{42}H_{69}NO_2$): $[M-H]^+$ 618.6, ^1H NMR (250 MHz, $CDCl_3$) was consistent with the expected benzoin product. To a solution of the benzoin product from above (24.7g, 40mmol) in dry toluene (500mL) was added sequentially 2-mercaptoethanol (25g, 320mmol) and TMSCl (100mL, 788mmol). The reaction solution was heated at reflux for 23 hr under argon before being cooled to room temperature. To this was added additional TMSCl (50mL, 394mmol); and the reaction solution was heated at reflux for another 3 hr. The resulting solution was cooled, was made basic with cold 2.5N aqueous NaOH and was extracted with CH_2Cl_2 (3x). The combined organic layers were washed with saturated aqueous $NaHCO_3$ (2x) and brine, was dried over Na_2SO_4 and was concentrated in vacuo to give a brown oily liquid. Preparative column chromatography on silica gel by using a Waters 500 Prep LC system (gradient elution with hexane, 1:99, 2:98 ethyl acetate:hexane) provided 15.5g (60%) of the C-28 thioxene as an orange-yellow oil (LSIMS ($C_{44}H_{71}NOS$): $[M-H]^+$ 661.6, ^1H NMR (250 MHz, $CDCl_3$) was consistent with the expected C-28 thioxene product 2-(4-(N,N-di-($C_{14}H_{29}$)-anilino)-3-phenyl thioxene.

Silicon tetra-t-butyl phthalocyanine:

Sodium metal, freshly cut (5.0g, 208mmol), was added to 300mL of anhydrous methanol in a two-liter, 3-necked flask equipped with a magnetic stirrer, reflux condenser, a drying tube and a gas bubbler. After the sodium was completely dissolved, 4-t-butyl-1,2-dicyanobenzene (38.64g, 210mmol, from TCI Chemicals, Portland OR) was added using a funnel. The mixture became clear and the temperature increased to about 50°C. At this point a continuous stream of anhydrous ammonia gas was introduced through the glass bubbler into the reaction mixture for 1 hr. The reaction mixture was then heated under reflux for 4 hr. while the stream of ammonia gas continued. During the course of the reaction, as solid started to precipitate. The resulting suspension was evaporated to dryness (house

vacuum) and the residue was suspended in water (400mL) and filtered. The solid was dried (60°C, house vacuum, P_2O_5). The yield of the product (1,3-diiminoisoindoline, 42.2g) was almost quantitative. This material was used for the next step without further purification. To a one-liter, three-necked flask equipped with a condenser and a drying tube was added the above product (18g, 89mmol) and quinoline (200mL, Aldrich Chemical Company, St. Louis MO). Silicon tetrachloride (11mL, 95mmol, Aldrich Chemical Company) was added with a syringe to the stirred solution over a period of 10 minutes. After the addition was completed, the reaction mixture was heated to 180-185°C in an oil bath for 1 hr. The reaction was allowed to cool to room temperature and concentrated HCl was carefully added to acidify the reaction mixture (pH 5-6). The dark brown reaction mixture was cooled and filtered. The solid was washed with 100mL of water and dried (house vacuum, 60°C, P_2O_5). The solid material was placed in a 1-liter, round bottom flask and concentrated sulfuric acid (500mL) was added with stirring. The mixture was stirred for 4 hr. at 60°C and was then carefully diluted with crushed ice (2000g). The resulting mixture was filtered and the solid wad washed with 100mL of water and dried. The dark blue solid was transferred to a 1-liter, round bottom flask, concentrated ammonia (500mL) was added, and the mixture was heated and stirred under reflux for 2 hr., was cooled to room temperature and was filtered. The solid was washed with 50mL of water and dried under vacuum (house vacuum, 60°C, P_2O_5) to give 12g of product silicon tetra-t-butyl phthalocyanine as a dark blue solid. 3-picoline (12g, from Aldrich Chemical Company), tri-n-butyl amine (anhydrous, 40mL) and tri-n-hexyl chlorosilane (11.5g) were added to 12g of the above product in a one-liter, three-necked flask, equipped with a magnetic stirrer and a reflux condenser. The mixture was heated under reflux for 1.5 hr. and then cooled to room temperature. The picoline was distilled off under high vacuum (oil pump at about 1mm Hg) to dryness. The residue was dissolved in CH_2Cl_2 and purified using a silica gel column (hexane) to give 10g of pure product di-(tri-n-hexylsilyl)-silicon tetra-t-butyl phthalocyanine as a dark blue solid. (LSIMS: $[M-H]^+$ 1364.2, absorption spectra: methanol: 674nm (ε 180,000): toluene 678nm,

^{1}H NMR (250 MHz, $CDCl_3$): δ: -2.4(m,12H), -1.3(m, 12H), 0.2-0.9 (m, 54H), 1.8(s, 36H), 8.3(d, 4H) and 9.6 (m, 8H) was consistent with the above expected product.

Hydroxypropylaminodextran: ($1NH_2$/ 7 glucose) was prepared by dissolving Dextran T-500 (Pharmacia, Uppsala, Sweden) (50g) in 150 mL of H_2O in a 3-neck round-bottom flask equipped with mechanical stirrer and dropping funnel. To the above solution was added 18.8g of Zn $(BF_4)_2$ and the temperature was brought to 87°C with a hot water bath. Epichlorohydrin (350mL) was added dropwise with stirring over about 30 min while the temperature was maintained at 87-88°C. The mixture was stirred for 4 hr while the temperature was maintained between 80°C and 95°C, then the mixture was cooled to room temperature. Chlorodextran product was precipitated by pouring slowly into 3L of methanol with vigorous stirring, recovered by filtration and dried overnight in a vacuum oven.

The chlorodextran product was dissolved in 200mL of water and added to 2L of concentrated aqueous ammonia (36%). This solution was stirred for 4 days at room temperature, then concentrated to about 190mL on a rotary evaporator. The concentrate was divided into two equal batches, and each batch was precipitated by pouring slowly into 2L of rapidly stirring methanol. The final product was recovered by filtration and dried under vacuum.

Hydroxypropylaminodextran ($1NH_2$/ 7 glucose), prepared above, was dissolved in 50mM MOPS, pH 7.2, at 12.5 mg/mL. The solution was stirred for 8 hr at room temperature, stored under refrigeration and centrifuged for 45 min at 15,000 rpm in a Sorvall RC-5B centrifuge immediately before use to remove a trace of solid material. To 10mL of this solution was added 23.1mg of Sulfo-SMCC in 1mL of water. This mixture was incubated for 1 hr at room temperature and used without further purification.

Chemiluminescer particles (TAR beads):

The following dye composition was employed: 20% C-28 thioxene (prepared as described above), 1.6%1-chloro-9,10-bis(phenylethynyl)anthracene (1-Cl-BPEA) (from Aldrich Chemical Company) and 2.7% rubrene (from (from Aldrich Chemical Company). The particles were latex particles (Seradyn Particle Technology,

Indianapolis IN). The dye composition (240-250 nM C-28 thioxene, 8-16 nM 1-Cl-BPEA, and 20-30 nM rubrene) was incorporated into the latex beads in a manner similar to that described in U.S. Patent 5,340,716 issued August 23, 1994 (the '716 patent), at column 48, lines 24-45, which is incorporated herein by reference. The dyeing process involved the addition of the latex beads (10% solids) into a mixture of ethylene glycol (65.4%), 2-ethoxyethanol (32.2%) and 0.1N NaOH (2.3%). The beads were mixed and heated for 40 minutes at 95°C with continuos stirring. While the beads are being heated, the three chemiluminescent dyes were dissolved in 2-ethoxyethanol by heating them to 95°C for 30 minutes with continuous stirring. At the end of both incubations, the dye solution was poured into the bead suspension and the resulting mixture was incubated for an additional 20 minutes with continuous stirring. Following the 20-minute incubation, the beads were removed form the oil bath and are allowed to cool to 40°C ± 10°C. The beads were then passed through a 43-micron mesh polyester filter and washed. The dyed particles were washed using a Microgon (Microgon Inc., Laguna Hills, CA). The beads were first washed with a solvent mixture composed of ethylene glycol and 2-ethoxyethanol (70%/30%). The beads were washed with 500 ml of solvent mixture per gram of beads. This is followed by a 10 % aqueous ethanol (pH 10-11) wash. The wash volume was 400 ml per gram of beads. The beads were then collected and tested for % solid, dye content, particle size, signal and background generation.

Oligonucleotide Bound Chemiluminescer Particles:

The oligonucleotide was immobilized on the surface of the above particles in the following manner. Aminodextran (500 mg) was partially maleimidated by reacting it with sulfo-SMCC (157 mg, 10 mL H_2O). The sulfo-SMCC was added to a solution of the aminodextran (in 40 mL, 0.05 M Na_2HPO_4, pH 7.5) and the resulting mixture was incubated for 1.5 hr. The reaction mixture was then dialyzed against MES/NaCl (2x2L, 10 mM MES, 10 mM NaCl, pH 6.0, 4°C). The maleimidated dextran was centrifuged at 15,000 rpm for 15 minutes and the supernatant collected. The supernatant dextran solution (54 mL) was then treated with imidazole (7 mL of 1.0 M solution) in MES buffer (pH 6.0) and into this stirred solution was added the

stained chemiluminescer particles (10 mL of 10mg/mL). After stirring for 10 minutes the suspension was treated with EDAC (7 mmol in 10 mM pH 6.0 MES) and the suspension stirred for 30 minutes. After this time, SurfactAmps® (Pierce) Tween-20 (10%, 0.780 mL) was added to the reaction mixture for a final concentration of 0.1%. The particles were then centrifuged at 15,000 rpm for 45 minutes and the supernatant discarded. The pellet was resuspended in MES/NaCl (pH 6.0, 10 mM, 100 mL) by sonication. Centrifugation at 15,000 rpm for 45 minutes, followed by pellet resuspension after discarding the supernatant, was performed twice. The maleimidated dextran chemiluminescer particles were stored in water as a 10 mg/mL suspension.

Thiolated oligonucleotide (oligonucleotide bearing a 5'-bis(6-hydroxyethyldisulfide) group) (Oligos Etc.) was dissolved in water at a concentration of 0.49 mM. To 116 μL of this solution was added 8.3 μL of 3.5 M sodium acetate, pH 5.3 and 8.9 μL of tris(carboxyethyl)phosphine (20 mM). After 30 minutes incubation at room temperature, 548 μL of cold ethanol was added and the mixture was maintained at about 20°C for 1.5 hour. The precipitated oligonucleotide was recovered by centrifugation for 2 min. at 15,000 rpm in an Eppendorf centrifuge, then dissolved in 37.5 μL of 5mM sodium phosphate, 2 mM EDTA, pH 6.

An aliquot of the maleimidated beads prepared above containing 22 mg beads was centrifuged for 30 min. at about 37,000 g, and the pellet was resuspended in 96 μL of 0.26 M NaCl, 0.05% Tween-20, 95 mM sodium phosphate, and 0.95 mM EDTA, pH7. The thiolated oligonucleotide was added and the mixture was maintained at 37°C for 64 hours under argon. A 10 μL aliquot of sodium thioglycolate was added and incubation was continued for 2 hours at 37°C. Water was added to a total volume of 1 mL, and the beads were recovered by centrifugation, then resuspended in 5 mL of 0.1 M NaCl, 0.17 M glycine, 10 mg/mL BSA, 1 mM EDTA, 0.1% Tween-20, and 0.5 mg/mL Calf thymus DNA (Sigma Molecular Biology grade), pH 9.2. After three hours, the beads were recovered and washed three times by centrifugation, twice in buffer A and once in standard PCR buffer. The product was stored refrigerated in PCR buffer. Buffer A contained 0.1 M

Tris base (J.T. Baker Chemical Co.), 0.3 M NaCl (Mallinckrodt), 25 mM EDTA Na_2 H_2O (Sigma Chemical Co.), 0.1% BSA (Sigma Chemical Co.), 0.1% dextran (Pharmacia), HBR-1 (Scantibodies), 0.05% Kathon and 0.01% gentamicin sulfate (GIBCO) prepared by dissolving and adjusting pH to 8.20 with concentrated HCl and made up to 10 L with distilled water.

The above procedure may be modified in a manner similar to that described by Ullman, *et al.*, Proc. Natl. Acad. Sci. USA (1994) 91:5426-5427 at column 1 of page 5427.

Sensitizer Particles:

Four mL of 20% suspension (400 mg) of washed 175 nm carboxylate modified latex was diluted with 3 mL of ethoxyethanol in a 25 mL round bottom (R.B.) flask with a stir bar. The R.B. flask was then placed in an oil bath at 105°C and stirred for 10 minutes. Then, 40 mg of silicon tetra-t-butyl phthalocyanine prepared as described above was added; the beads were stirred for 5 minutes more. At this point 1.0 mL of 0.1N NaOH was added slowly over 5 minutes. During all the additions, the oil bath temperature was maintained at 105°C. The oil bath temperature was slowly allowed to drop to room temperature over 2 hours. After cooling, the mixture was diluted with 20 mL of ethanol and centrifuged (12,500 rpm, 30 minutes). Supernatants were discarded and the pellets resuspended in ethanol by sonication. Centrifugation was repeated, and the pellet was resuspended in water; and centrifugation was repeated. The pellet was resuspended in 5 mL of aqueous ethanol to a final volume of 40 mL.

Streptavidin was bound to the above beads using 25mg streptavidin for 100mg of beads. 25mg streptavidin (50mg Aaston solid from Aaston, Wellesley, MA) was dissolved in 1mL of 1mM EDTA, pH 7.5, and 77μL of 2.5mg/mL SATA in ethanol was added thereto. The mixture was incubated for 30 min at room temperature. A deacetylation solution was prepared containing 1M hydroxylamine-HCl, 50mM Na_2PO_4, 25mM EDTA, pH 7.0. 0.1mL of this deacetylation solution was added to the above solution and incubated for 1 hr at room temperature. The resulting thiolated streptavidin was purified on a Pharmacia PD10 column and washed with a column buffer containing 50mM MOPS, 50mM EDTA, pH 7.2. The

volume of the sample was brought to 2.5mL by adding 1.5mL of the above column buffer. The sample was loaded on the column and eluted with 3.5mL of the column buffer. The thiolated streptavidin was diluted to 5mL by adding 1.5mL of 50mM MOPS, 50mM EDTA, 0.1% Tween-20, pH 7.2. 5mL of the thiolated streptavidin solution was added to 5mL of maleimidated sensitizer beads prepared as described above, under argon, and mixed well. The beads were topped with argon for 1 min, the tube was sealed and the reaction mixture was incubated overnight at room temperature in the dark.

To the above beads was added 7.5mL of 50mM MOPS, 50mM EDTA, 0.1% Tween-20, pH 7.2 to bring the beads to 1mg/mL. The remaining maleimides were capped by adding mercaptoacetic acid at a final concentration of 2mM. The mixture was incubated in the dark for 30 min at room temperature. The remaining thiols were capped by adding iodoacetic acid at a final concentration of 10mM and the mixture was incubated at room temperature for 30 min in the dark. The beads were centrifuged for 30 min at 15,000 rpm as above for a total of three times.

Oligonucleotide bound Sensitizer Particles:

Oligonucleotides were bound to the sensitizer particles prepared as described above by employing biotinylated oligonucleotides. This procedure was as follows: biotinylated oligonucleotides (from Oligos Etc.) were mixed with streptavidin beads prepared as described above in a ratio of 0.5 to 0.75 oligonucleotides per streptavidin bead in the NL buffer (10 mM Tris HCl, 70 mM KCl, 12 mM $MgCl_2$, 0.2 mg/ml acetylated BSA, pH 8.0)

Preparation of a soluble Sensitizer-Oligonucleotide Conjugate:

A sensitizer-BSA conjugate was obtained from Ultra Diagnostics Corporation, Seattle, Washington, and described by them as UT680-BSA-SPDP. This material carries about 4.5 sensitizers, namely, aluminum tetrabenztriazaporphyrin sulfonates as described in WO 90/02747, Example 4, per BSA molecule, as well as pyridyldisulfide substituents to allow chemical modification. Concentration was approx. 3 mg/mL in 0.2M aqueous $NaHCO_3$.

To 1.4 mL UT680 (4.2 mg) was added 0.28 mL of 50 mM DTT in 0.5 M Na_2HPO_4, pH 7.0. After standing 1 hr at room temperature, an aliquot of this

material was diluted 1:50 in 0.1 M Na_2HPO_4, pH 7.0 and its UV-visible spectrum was recorded. The amount of thiopyridone released was calculated by comparing the ratio of absorbance at 342 nm (thiopyridone) and 678 nm (sensitizer) to the ratio obtained from the starting material. Using ε_{342}= 7200 for thiopyridone, the ratio of thiopyridone released to BSA was found to be 4.5.

DTT was removed from the reduced material on a PD-10 column (Pharmacia) using 10 mM Na_2HPO_4, 1 mM EDTA, pH 7.0. Fractions containing green color (2.5 mL) were combined. DMSO (0.95 mL) followed by 200 μL of 25 mM Bismaleimidohexane (Pierce) in DMSO was added to the combined fractions. After 15 minutes at room temperature, a second aliquot of Bismaleimidohexane was added – some cloudiness in the reaction was noted at this point. After 30 minutes more, the product was dialyzed cold overnight in the dark against 1 L of 5 mM Na_2HPO_4, 1 mM EDTA, pH 6.0, then cold for 24 hours against 1 L of 5 mM NaCl. The recovered crude product was centrifuged for 10 minutes to remove a trace of precipitate.

The amount of maleimide incorporated in the UT- 680 was estimated by reacting an aliquot with 25 μM cysteine in 60 mM Tris, 6 mM EDTA, pH 7.8 bubbled with argon. After 30 minutes at room temperature, 10 μL of a 4 mg/mL solution of 5,5'- dithio-bis(2-nitrobenzoic acid) (DTNB) in ethanol was added. The solution was incubated 10 minutes more, and absorbance was read at 412 and 678 nm. Suitable controls were run omitting either cysteine or the product. Maleimide content was calculated from the amount of cysteine depleted from the solution. Cysteine was determined from absorbance at 412 nm. The absorbance in controls containing cysteine only corresponded very closely with that predicted using the literature extinction coefficient of 13,600 for the DTNB adduct of cysteine. Since the UT- 680 has considerable absorbance at 412 nm, a correction was made to the measured A_{412} for the reaction with cysteine. The cysteine depleted corresponded to about 5.5 maleimides per BSA.

An oligonucleotide with the following sequence was purchased from Oligos Etc. Inc.:

5'-CCATCAATGAGGAAGCTGCAGAATAGAAGGAAGTCTTTX-3' SEQ ID NO: 9 where X is a 3' disulfide modifier $OPO_2^-O(CH_2)_3$-SS-$(CH_2)_3OH$. The HPLC-purified product was dissolved in 108 μL of water. It's concentration was 6.85×10^{-4}M determined by absorbance at 260 nm of a diluted aliquot. A 43.8 μL portion of this oligonucleotide (30nmoles) was mixed with 3.2 μL of 3.5 M acetate pH 5.3 and 7.7 μL of tris(carboxyethyl) phosphine hydrochloride (5.73 mg/mL in water). After 1 hour incubation at room temperature, 219 μL of cold ethanol was added and the mixture was stored for 2 hours at -20°C. The precipitate was recovered by 5 minutes centrifugation in an Eppendorf centrifuge at 14,000 rpm, then redissolved in 31.8 μL of water and 2.3 μL of acetate and re-precipitated with 136 μL of ethanol. After 2 more hours at -20°C and centrifugation as above, the pellet was dissolved in 42.3 μL of water. A 2 μL aliquot was removed and frozen for later analysis.

The maleimidated UT-680 solution described above was concentrated to about 985 μL using Centricon concentrators, then mixed with 20 μL of 5 M NaCl, 100 μL of 0.4 M Na_2HPO_4 pH 7.0, and 10 μL of 0.1 M disodium EDTA. The reduced oligonucleotide was added, and the mixture was transferred to a 15 mL conical centrifuge tube and saturated with argon. This solution was incubated for approximately 64 hours at room temperature inside a second tube also filled with argon.

Maleimides in the crude product were capped by adding 11.2 μL of 0.1 M $HSCH_2COO^-Na^+$ in water and incubating for 45 minutes at room temperature. The product was dialyzed cold dark overnight against 1 L of 10 mM NaCl, then concentrated to ca. 0.5 mL using a Centricon 30 and stored refrigerated. Analysis of this solution by reverse-phase HPLC showed a mixture of starting oligonucleotide, the oligonucleotide dimer, and the conjugate. Individual peaks were identified by collection and determination of their absorbance spectrum, comparing the ratio of absorbance at 260 nm from oligonucleotide to 678 nM from sensitizer. Quantitative recovery from HPLC was not good enough to allow a practical purification by this route.

Approximately one third of the crude product was purified by diluting to 0.5 mL with water, adding 5M NaCl to give 0.1 M final concentration, and concentrating to 50-100 μL by centrifugation for about 11 minutes in a Centricon 100. The concentrate was rediluted to 5 mL with 0.1 M NaCl, concentrated again to 50-100 μL, and recovered from the concentrator using water washes to give a final volume of ca. 250 μL. (Previous experiments showed that this treatment removed free oligonucleotide to give a constant 260/678 nm absorbance ratio).

A Bio-Rad disposable column was packed with 2 mL of DEAE Sepharose Fast Flow (Pharmacia) and washed with 20 mM Tris pH 8.0 (approximate column dimensions 1 cm x 4cm). The concentrate above was added to the column and eluted as follows: 3 mL 20 mM Tris, pH 8; 3 mL Tris + 0.3 M NaCl; 3 mL Tris + 0.4 mL NaCl; 3 mL Tris + 0.5 M NaCl; 3 mL Tris + 1 M NaCl; and 2 mL Tris + 3 M NaCl. Fractions (ca. 0.36 mL) were diluted to 0.5 mL and analyzed by absorbance. Two major peaks were obtained. The peak centered at 8.25 mL eluate had a ratio of A_{260}/A_{678}= 0.77, similar to the starting sensitizer. A second peak centered at 11.25 mL had a ratio of 1.21 and was identified as conjugate. This solution was stored refrigerated in the dark. Analysis on a Novex polyacrylamide non-denaturing gradient gel (4-20%) showed some free oligonucleotide and dimer stained with Cyber Green ™ and a new band stained with both Cyber Green and Coomassie Blue (presumably conjugate).

Example 1

In the following example a first oligonucleotide probe was attached to chemiluminescer particles as described above. A second oligonucleotide probe was a 3'-biotin labeled oligonucleotide (from Oligos Etc., Inc., Wilsonville, OR) and was composed of two parts. The 3' part was a 23 nucleotide long sequence derived from an HIV target polynucleotide (described in GenBank accession no. AF033819 HIV-1 complete genome). The 5'portion was either a 10 (AN1) or 12 (AN2) nucleotide long sequence complementary to the sequence immobilized on the

chemiluminescer particle. The target polynucleotide was composed of two parts: The 5'-portion was derived form the HIV target polynucleotide mentioned above and was complementary to the respective sequence on the biotin labeled probe, and the 3'-portion was complementary to a sequence of the oligonucleotide probe immobilized on the chemiluminescer particle.

The signal was generated by the association of the oligonucleotide chemiluminescer particle and a streptavidin sensitizer particle bound to the biotin labeled oligonucleotide probes. Hybridization of the two oligonucleotide probes was not sufficient for forming a stable association. The formation of a stable complex of association of the two particles and generation of a signal was dependent on the cooperative binding of the two probes and the target polynucleotide.

The following oligonucleotide probes were employed. All oligonucleotide probes were from Oligos Etc. Inc.

Probes bound to the sensitizer particles:

AN1 3'-biotinGACCTACGTGAGATAGGGTAAGGTCATTCATTC (SEQ ID NO:1)

AN2 3'-biotinGACCTACGTGAGATAGGGTAAGGTCATTCATTCAT (SEQ ID NO:2)

Probes immobilized on the chemiluminescer particles:

3'$(ATGA)_6$ (SEQ ID NO:3)

Target polynucleotides:

AN3 5'CTGGATGCACTCTATCCCATTCGTACTTACTTA (SEQ ID NO:4)

AN4 5' CTGGATGCACTCTATCCCATTCGTACTTACTTACT (SEQ ID NO:5)

The underlined sequences were derived form the target HIV. The underlined sequence in the AN1 and AN2 oligonucleotide probes were complementary to the underlined sequence in the target AN3 and AN4. The AN1 and AN2 oligonucleotide probes differ by two additional nucleotides in the last position on AN2 to provide a 10 or 12 base long complementary with the chemiluminescer immobilized oligonucleotide probe. The AN3 and AN4 target molecules are similarly different

from each other. The G nucleotide immediately after the underlining is a spacer between the two described parts of the oligonucleotide probes.

Oligonucleotide probes AN1 or AN2 were bound to streptavidin sensitizer particles to an average 0.5 oligonucleotide load per particle. Oligonucleotide sensitizer particles were mixed with chemiluminescer oligonucleotide particles in NL buffer (10mM Tris-HCl, 70mM KCl, 12 mM $MgCl_2$, pH 8.0 acetylated BSA 0.2 mg/ml). The bead mixture (1 μg per assay tube) were added to a solution containing no target or targets AN3 or AN4 (0.2 femtomole per reaction). The reaction mixture (50 μl) were covered with a drop of oil (mineral oil, white, Aldrich Chemical Co.) and incubated as follows: 2 min. at 50°C (denaturation), 30 min. at37°C. Signal was determined by illumination for 0.1 sec. and read for 1 sec. The results are summarized in Table 2.

Table 2

Probes	Targets		
	None	AN3	AN4
Chemiluminescer only	194	192	214
AN1 + chemiluminescer	214	528	2128
AN2 + chemiluminescer	646	3390	6328

The above results demonstrate that signal is dependent on the presence of the target polynucleotide and is generated by the cooperativity of binding of the chemiluminescer probe to the target and the second probe, AN1 or AN2, respectively.

Example 2

The oligonucleotide probes and the target molecules are the same as those used above in Example 1. Chemiluminescer oligonucleotide particles and streptavidin oligonucleotide probe AN1 or AN2 (1.25 μg) were mixed with target molecule, AN3 or AN4, in NL buffer to a total volume of 50 μl per assay. The

reaction mixture was covered with a drop of oil and incubated as follows: 2 min. at 50°C (denaturation), 30 min. at 38°C. Signal was determined by illumination for 0.1 sec. and read for 1 sec. Target concentrations were 0, 2, 20 and 200 nM. The results are summarized in Table 3.

Table 3

Targets	Target concentration	Probes		
		None	AN1	AN2
None		282	262	344
AN3	2 nM	248	330	1106
AN4	2 nM	268	796	3384
AN3	20 nM	316	684	4200
AN4	20 nM	286	4204	14850
AN3	200 nM	586	1910	7646
AN4	200 nM	930	9870	26668

The highest signal response to target was with target and oligonucleotides that provided 12-nucleotide complementarity to the hybridization of he chemiluminescer probe with both the target molecule and the second oligonucleotide probe. The response was target concentration dependent.

The above results demonstrate that signal is proportional to the concentration of the target polynucleotide.

Example 3

Evaluation of different oligonucleotides P_T with different lengths to detect amplified HIV-1 RNA

Oligonucleotides P_T contained three specific DNA regions that were capable of binding to:

1. a nucleic acid target

2. chemiluminescer beads containing oligonucleotides with a complementary sequence
3. a universal probe P consisting of soluble sensitizer conjugate coupled to a oligonucleotide with a complementary sequence P3 prepared as described above.

Different oligonucleotides P_T with different lengths complementary to a HIV-1 specific target sequence were evaluated; for example, oligonucleotide P_T9 contains 9 nucleotides complementary to 9 nucleotides of HIV-1 RNA, oligonucleotide P_T11 contains 11 nucleotides complementary to 11 nucleotides of HIV-1 RNA, etc.

A reaction mixture containing 2 μL amplified HIV-1 RNA (wildtype, 2.4×10^{11} copies/reaction amplified by NASBA) or 2 μL water, different oligonucleotides P_T (62.5 nM), buffer 50 mM KCl, 4mM Mg_2Cl, 10 mM Tris-HCl pH 8.3 and 200μg/ml BSA) and a soluble sensitizer conjugate (500 pM) were incubated for 3 minutes at 65°C, 10 minutes at 50°C and for 60 minutes at 41°C. Chemiluminescer particles (1.25 μg) and buffer (50 mM KCl, 4 mM Mg_2Cl, 10 mM Tris-HCl pH 8.3 and 200 μg/ml BSA) were added and incubated for 60 minutes at 41°C. The final volume of the reaction was 20 μl. The individual samples were illuminated for one second and the emitted light was read for one second employing an instrument for reading the signal produced. This cycle was repeated three times and the incubation temperature was 41°C.

HIV-1 RNA was detected by using oligonucleotides P_T, which contained a varying lengths of nucleotides complementary to the target sequence. In this system length of the nucleotide sequence complementary to the target had an effect on the results:

- Oligonucleotides P_T containing less than 13 nucleotides complementary to the target sequence showed no positive/negative discrimination due to their inability to form a stable structure, i.e. P_T9 and P_T11.
- Oligonucleotides P_T containing at least 13 nucleotides complementary to the target sequence formed a stable structure due to cooperative binding effects and allowed a positive/negative discrimination, e.g. P_T13 and P_T15.

The oligonucleotides used were as follows:

PT13: 5'-$(TACT)_5$CTCTTCCTTCAAAATGGGATAGAGTG –3' (SEQ ID NO:6) (EB2)

PT15: 5'-$(TACT)_5$CTCTTCCTTCAAAATGGGATAGAGTGCA –3' (SEQ ID NO:7) (EB3)

HIV RNA: 3'-GGUAGUUACUCCUUCGACGUCUU-ACCCUAUCUCACGUAG-5' (SEQ ID NO:8) (WT)

Sensitizer oligonucleotide:

5'-CCATCAATGAGGAAGCTGCAGAATAGAAGGAAGTCTTT-3' (SEQ ID NO:9) wherein the underlined portions were designated P2 and P3, respectively, and sensitizer is attached at the 3'end through a BSA linker

Chemiluminescer oligonucleotide:

3'-ACGTGAGATAGGGTAAAACTTCCTTCTC$(TCAT)_5$ (SEQ ID NO:10) wherein the underlined portion is complementary to the underlined sequence in the following oligonucleotide to which a chemiluminescer particle is attached at the 5'-end:

3'-$(AGTA)_5$-AGTA-5' (SEQ ID NO:11)

The above system is depicted in Fig. 6 wherein CL refers to chemiluminescer incorporated in the latex particle, S refers to sensitizer, which is attached to the oligonucleotide through a BSA linker. The results are summarized in Fig. 7.

The above discussion includes certain theories as to mechanisms involved in the present invention. These theories should not be construed to limit the present invention in any way, since it has been demonstrated that the present invention achieves the results described.

The above description and examples fully disclose the invention including preferred embodiments thereof. Modifications of the methods described that are obvious to those of ordinary skill in the art such as molecular biology and related sciences are intended to be within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A method for detecting a single stranded target polynucleotide, said method comprising:

forming a ternary complex comprising said target polynucleotide and first and second oligonucleotide probes, wherein said first and second oligonucleotide probes respectively have different first sequences that are complementary to said target polynucleotide and different second sequences that are complementary to each other, under conditions wherein said first and second oligonucleotide probes do not substantially bind to each other in the absence of said target polynucleotide and

detecting the association of said first and second oligonucleotide probes.

2. A method for determining the amount of a target polynucleotide, said method comprising:

forming (1) a first ternary complex comprising said target polynucleotide and first and second oligonucleotide probes wherein said first and second oligonucleotide probes respectively have different first sequences that are complementary to said target polynucleotide and different second sequences that are complementary to each other and (2) a second ternary complex comprising a reference polynucleotide, said first oligonucleotide probe, and a third oligonucleotide probe, wherein said reference polynucleotide has a sequence that is complementary to said first sequence of said first oligonucleotide probe and said third oligonucleotide probe has a first sequence that is complementary to said reference polynucleotide but not to said target polynucleotide and a second sequence that is identical to the second sequence of said second oligonucleotide probe, wherein the conditions for forming said ternary complexes are controlled to avoid substantial binding of said first oligonucleotide probe with said second and third oligonucleotide probes in the absence of said target and said reference polynucleotide probes, and

determining the ratio of said ternary complexes and relating said ratio to the amount of said target polynucleotide.

3. The method of Claim 2 wherein each of said ternary complexes is capable of producing a signal and the ratio of said signal produced by said ternary complexes is determined and related to the amount of said target polynucleotide.

4. A method for detecting a single stranded target polynucleotide, which comprises:

(a) providing in combination (i) a medium suspected of containing said target polynucleotide having a sequence T2, (ii) a reference polynucleotide having a sequence R2 that is identical to T2 and a sequence R1 that is not present in said target polynucleotide, (iii) a first oligonucleotide probe capable of independently hybridizing to T2 and R2, (iv) a second oligonucleotide probe that binds to a duplex TU of said target polynucleotide and said first oligonucleotide probe but not to said target polynucleotide or said first oligonucleotide probe apart from TU, and (v) a third oligonucleotide probe that binds to a duplex RU of said reference polynucleotide and said first oligonucleotide probe but not to said reference polynucleotide or said first oligonucleotide probe apart from RU,

(b) incubating said combination under conditions such that said target polynucleotide and said reference polynucleotide can bind, respectively, to said first oligonucleotide probe to form TU and RU and said second and third oligonucleotide probes can bind respectively to TU and RU to form ternary complexes, and

(c) determining the ratio of said ternary complexes and relating said ratio to the concentration of said target polynucleotide in said medium.

5. The method of Claim 4 wherein said second oligonucleotide probe and said third oligonucleotide probe each comprise a member of a different signal producing system and the signals measured from said ternary complexes are used to determine said ratio.

6. The method of Claim 5 wherein said first oligonucleotide probe comprises a member of both signal producing systems.

7. A method for determining the amount of a single stranded target polynucleotide present in a medium, which comprises:

(a) providing in combination (i) a measured amount of a medium suspected of containing said single stranded target polynucleotide, said single stranded target polynucleotide comprising a sequence T1 and a sequence T2, (ii) a predetermined amount of a reference polynucleotide comprising a sequence R2 that is identical to T2 and a sequence R1 that is not present in said target polynucleotide, (iii) a first oligonucleotide probe comprising a first sequence P2 capable of hybridizing to R2 and T2 and a sequence P3 that is not capable of hybridizing to said target polynucleotide or said reference polynucleotide, (iv) a second oligonucleotide probe comprising a sequence PT1 capable of hybridizing to T1 and a sequence PT3 that is capable of hybridizing to P3, and (v) a third oligonucleotide probe comprising a sequence PR1 that is capable of hybridizing to R1 and a sequence PR3 that is identical to PT3 and is capable of hybridizing to P3, wherein R1, T1, P3, PR1, PR3, PT1 and PT3 are constructed such that respective hybridizable pairs thereof do not form stable duplexes under the conditions in step (b) below,

(b) incubating said combination under conditions such that said target polynucleotide and said reference polynucleotide bind, respectively, to said first oligonucleotide probe to form independent duplexes TU and RU and said second and third oligonucleotide probes bind respectively to TU and RU to form ternary complexes, and

(c) determining the ratio of said ternary complexes, and

(d) relating said ratio to the amount of said single stranded target polynucleotide in said medium.

8. The method of Claim 7 wherein said second oligonucleotide probe and said third oligonucleotide probe each comprise a member of a different signal producing system and the signals measured from said ternary complexes are used to determine said ratio.

9. The method of Claim 8 wherein said first oligonucleotide probe comprises a member of both signal producing systems.

10. The method of Claim 7 wherein said incubation is carried out at an isothermal temperature.

11. The method of Claim 8 wherein the concentration of said target polynucleotide in said medium increases during said method and a change in said signal is monitored at one or more points in time during said method to determine the concentration of said target polynucleotide.

12. A method for quantitating a target polynucleotide by amplifying and detecting a product of the amplification of said target polynucleotide comprised of sequences T1 and T2, said method comprising:

(a) providing in combination (i) a medium suspected of containing said target polynucleotide, said medium having a measured concentration of a reference polynucleotide that yields a reference amplification product comprising a sequence R2 that is identical to said sequence T2, and a sequence R1 that is different from said sequence T1, (ii) all reagents required for conducting an amplification of said target polynucleotide and said reference polynucleotide to produce amplification products of said target polynucleotide and said reference polynucleotide, (iii) a first oligonucleotide probe comprising a first sequence P2 capable of hybridizing to R2 and T2 and a sequence P3 that is not capable of hybridizing to the amplification product of said target polynucleotide or the amplification product of said reference polynucleotide, (iv) a second oligonucleotide probe comprising a sequence PT1 capable of hybridizing to T1 and a sequence PT3 that is capable of hybridizing to P3, and (v) a third oligonucleotide probe comprising a sequence PR1 that is capable of hybridizing to R1 and a sequence PR3 that is identical to PT3 and is capable of hybridizing to P3, wherein said sequences R1, T1, P3, PR1, PR3, PT1 and PT3 are constructed such that respective hybridizable pairs thereof do not form stable duplexes under the conditions in step (b) below,

(b) incubating said combination under conditions such that the amplification products of said target polynucleotide and said reference polynucleotide are formed and bind, respectively, to said first oligonucleotide probe to form independent duplexes TU and RU and said second and third oligonucleotide probes bind respectively to TU and RU to form ternary complexes,

(c) determining the ratio of said ternary complexes, and

(d) relating said ratio to the concentration of said target polynucleotide in said medium during said amplification.

13. The method of Claim 12 wherein said incubation is carried out at an isothermal temperature.

14. The method of Claim 12 wherein said second oligonucleotide probe and said third oligonucleotide probe each comprise a member of a different signal producing system and the signals measured from said ternary complexes are used to determine said ratio.

15. The method of Claim 14 wherein said first oligonucleotide probe comprises a member of both signal producing systems.

16. The method of Claim 12 wherein said first oligonucleotide probe in combination with each of said second oligonucleotide probe and said third oligonucleotide probe comprise members of different signal producing systems and the signals measured from said ternary complexes are used to determine said ratio.

17. The method of Claim 12 wherein said first oligonucleotide probe comprises a first member of each of two signal producing systems and wherein said second oligonucleotide probe and said third oligonucleotide probe each respectively comprise a second member of each of said signal producing systems.

18. The method of Claim 17 wherein, when said first member is brought into close proximity with said second members, a signal is produced.

19. The method of Claim 18 wherein said first member is an enzyme and said second members are enzymes that are different from the first enzyme and from each other and the products of the reaction of the enzyme comprising the first member are the substrates for the other of said enzymes.

20. The method of Claim 18 wherein said first member is a sensitizer and said second members are chemiluminescent compounds that emit at different wavelengths or with different decay rates.

21. The method of Claim 18 wherein said first member is a chemiluminescent compound and said second members are sensitizers that can be independently excited by different wavelengths of light.

22. The method of Claim 18 wherein said first member is an energy donor or acceptor and said second members are fluorescent compounds that emit at different wavelengths or with different decay rates.

23. The method of Claim 12 wherein said amplification is carried out at an isothermal temperature and a change in signal ratio is monitored during said amplification to determine the concentration of said amplification product.

24. The method of Claim 12 wherein said amplification is selected from the group consisting of PCR, NASBA, 3SR, SDA and amplifications utilizing Qβ-replicase.

25. The method of Claim 12 wherein said target polynucleotide is DNA.

26. The method of Claim 12 wherein said target polynucleotide is RNA.

27. The method of Claim 12 wherein a plurality of reference polynucleotides is employed in predetermined amounts.

28. The method of Claim 12 wherein the lengths of R1, T1, P3, PR1, PR3, PT1 and PT3 are each independently 8 to 16 nucleotides.

29. A method for quantitating a target RNA by amplifying and detecting the amplification products of said target RNA comprised of sequences T1 and T2, which comprises:

(a) providing in combination (i) a medium suspected of containing said target RNA, said medium having a measured concentration of a reference polynucleotide that yields a reference amplification product comprising a sequence R2 that is identical to said sequence T2, and a sequence R1 that is different from said sequence T1, (ii) all reagents required for conducting an amplification of said target RNA and said reference polynucleotide by NASBA to produce an amplification products of said target RNA and said reference polynucleotide, (iii) a first oligonucleotide probe comprising a first sequence P2 capable of hybridizing to R2 and T2 and a sequence P3 that is not capable of hybridizing to said amplification products of said target RNA or said reference polynucleotide, (iv) a second oligonucleotide probe comprising a sequence PT1 capable of hybridizing to T1 and a sequence PT3 that is capable of hybridizing to P3, and (v) a third oligonucleotide probe comprising a sequence PR1 that is capable of hybridizing to R1 and a sequence PR3 that is identical to PT3 and is capable of hybridizing to P3, wherein said sequences R1, T1, P3, PR1, PR3, PT1 and PT3 are constructed such that respective hybridizable pairs thereof do not form stable duplexes under the conditions in step (b) below,

(b) incubating said combination under conditions such that amplification products of said target RNA and said reference polynucleotide are formed and bind, respectively, to said first oligonucleotide probe to form independent duplexes TU and RU and said second and third oligonucleotide probes bind respectively to TU and RU to form ternary complexes,

(c) determining the ratio of said ternary complexes, and

(d) relating said ratio to the concentration of said target RNA in said medium during said amplification.

30. The method of Claim 29 wherein said second oligonucleotide probe and said third oligonucleotide probe each comprise a member of a different signal producing system and the signals measured from said ternary complexes are used to determine said ratio.

31. The method of Claim 30 wherein said first oligonucleotide probe comprises a member of both signal producing systems.

32. The method of Claim 29 wherein said first oligonucleotide probe in combination with each of said second oligonucleotide probe and said third oligonucleotide probe comprise members of different signal producing systems and the signals measured from said ternary complexes are used to determine said ratio.

33. The method of Claim 29 wherein said first oligonucleotide probe comprises a first member of each of two signal producing systems and wherein said second oligonucleotide probe and said third oligonucleotide probe each respectively comprise a second member of each of said signal producing systems.

34. The method of Claim 33 wherein, when said first member is brought into close proximity with said second members, a signal is produced.

35. The method of Claim 34 wherein said first member is an enzyme and said second members are enzymes that are different from the first enzyme and from each other and the products of the reaction of the enzyme comprising the first member are the substrates for the other of said enzymes.

36. The method of Claim 34 wherein said first member is a sensitizer and said second members are chemiluminescent compounds that emit at different wavelengths or with different decay rates.

37. The method of Claim 34 wherein said first member is a chemiluminescent compound and said second members are sensitizers that can be independently excited by different wavelengths of light.

38. The method of Claim 34 wherein said first member is an energy donor or acceptor and said second members are fluorescent compounds that emit at different wavelengths or with different decay rates.

39. The method of Claim 29 wherein a change in signal ratio is monitored during said amplification to determine the concentration of said amplification product.

40. The method of Claim 29 wherein a plurality of reference polynucleotides is employed in predetermined amounts.

41. The method of Claim 29 wherein the lengths of R1, T1, P3, PR1, PR3, PT1 and PT3 are each independently 8 to 16 nucleotides.

42. A kit for use in detection of a target polynucleotide comprising sequences T1 and T2, said kit comprising in packaged combination:

(a) a reference polynucleotide comprising a sequence R2 that is identical to T2 and a sequence R1 that is not present in said target polynucleotide,

(b) a first oligonucleotide probe comprising a first sequence P2 capable of hybridizing to R2 and T2 and a sequence P3 that is not capable of hybridizing to said target polynucleotide or said reference polynucleotide,

(c) a second oligonucleotide probe comprising a sequence PT1 capable of hybridizing to T1 and a sequence PT3 that is capable of hybridizing to P3, and

(d) a third oligonucleotide probe comprising a sequence PR1 that is capable of hybridizing to R1 and a sequence PR3 that is identical to and is capable of hybridizing to P3,

wherein the lengths of R1, T1, P3, PR1, PR3, PT1 and PT3 are such that respective hybridizable pairs thereof do not form stable duplexes under conditions for formation of a stable ternary complex of said target polynucleotide with said first and second oligonucleotide probes.

43. The kit of Claim 42 wherein said second oligonucleotide probe and said third oligonucleotide probe each comprise a member of a different signal producing system and the signals measured from said ternary complexes are used to determine said ratio.

44. The kit of Claim 43 wherein said first oligonucleotide probe comprises a member of both signal producing systems.

45. The kit of Claim 42 wherein said first oligonucleotide probe comprises a first member of a each of two signal producing systems and wherein said second oligonucleotide probe and said third oligonucleotide probe each respectively comprise a second member of each of said signal producing systems.

46. The kit of Claim 42 wherein, when said first member is brought into close proximity with said second members, a signal is produced.

47. The kit of Claim 46 wherein said first member is an enzyme and said second members are enzymes that are different from the first enzyme and from each other and the products of the reaction of the enzyme comprising the first member are the substrates for the other of said enzymes.

48. The kit of Claim 46 wherein said first member is a sensitizer and said second members are chemiluminescent compounds that emit at different wavelengths or with different decay rates.

49. The method of Claim 46 wherein said first member is a chemiluminescent compound and said second members are sensitizers that can be independently excited by different wavelengths of light.

50. The kit of Claim 46 wherein said first member is an energy donor or acceptor and said second members are fluorescent compounds that emit at different wavelengths or with different decay rates.

51. The kit of Claim 42 wherein said reagents for conducting an amplification are those for an amplification selected from the group consisting of PCR, NASBA, 3SR, SDA and amplifications utilizing Qβ-replicase.

52. The kit of Claim 42 wherein said target polynucleotide is DNA.

53. The kit of Claim 42 wherein said target polynucleotide is RNA.

54. The kit of Claim 42 which comprises a plurality of reference polynucleotides in predetermined amounts based on a suspected concentration of said target polynucleotide.

55. A kit for use in quantitating a target RNA by amplifying and detecting amplification products of said target RNA comprising sequences T1 and T2, said kit comprising in packaged combination:

(a) a promoter,

(b) an RNA polymerase,

(c) a reference polynucleotide comprising a sequence R2 that is identical to T2 and a sequence R1 that is different from said sequence T1,

(d) a first oligonucleotide probe comprising a first sequence P2 capable of hybridizing to R2 and T2 and a sequence P3 that is not capable of hybridizing to said target RNA or said reference polynucleotide,

(e) a second oligonucleotide probe comprising a sequence PT1 capable of hybridizing to T1 and a sequence PT3 that is capable of hybridizing to P3, and

(f) a third oligonucleotide probe comprising a sequence PR1 that is capable of hybridizing to R1 and a sequence PR3 that is identical to PT3 and is capable of hybridizing to P3,

wherein the lengths of R1, T1, P3, PR1, PR3, PT1 and PT3 are such that respective hybridizable pairs thereof do not form stable duplexes under the conditions for formation of a stable ternary complex of said amplification product of said target RNA with said first and second oligonucleotide probes.

56. The kit of Claim 55 wherein said second oligonucleotide probe and said third oligonucleotide probe each comprise a member of a different signal producing system and the signals measured from said ternary complexes are used to determine said ratio.

57. The kit of Claim 55 wherein said first oligonucleotide probe comprises a member of both signal producing systems.

58. The kit of Claim 55 wherein said first oligonucleotide probe comprises a first member of a each of two signal producing systems and wherein said second oligonucleotide probe and said third oligonucleotide probe each respectively comprise a second member of each of said signal producing systems.

59. The kit of Claim 58 wherein, when said first member is brought into close proximity with said second members, a signal is produced.

60. The kit of Claim 58 wherein said first member is an enzyme and said second members are enzymes that are different from the first enzyme and from

each other and the products of the reaction of the enzyme comprising the first member are the substrates for the other of said enzymes.

61. The kit of Claim 58 wherein said first member is a sensitizer and said second members are chemiluminescent compounds that emit at different wavelengths or with different decay rates.

62. The method of Claim 58 wherein said first member is a sensitizer and said second members are chemiluminescent compounds that emit at different wavelengths or with different decay rates.

63. The method of Claim 58 wherein said first member is a chemiluminescent compound and said second members are sensitizers that can be independently excited by different wavelengths of light.

64. The kit of Claim 58 wherein said first member is an energy donor or acceptor and said second members are fluorescent compounds that emit at different wavelengths or with different decay rates.

65. The kit of Claim 55 which comprises a plurality of reference polynucleotides in predetermined amounts.

66. A reagent for detecting a target polynucleotide, said reagent comprising two oligonucleotide probes capable of binding to a single strand of said target polynucleotide wherein said first and second oligonucleotide probes respectively have different first sequences that are complementary to said target polynucleotide and different second sequences that are complementary to each other and 8 to 16 nucleotides in length.

67. A kit for detection of a single stranded target polynucleotide, said kit comprising in packaged combination first and second oligonucleotide probes having

respectively different first sequences that are both complementary to said single stranded target polynucleotide and different second sequences that are complementary to each other and 8 to 16 nucleotides in length.

68. The method of Claim 18 wherein said first member is a fluorescent compound and said second members are energy donors that have excited states with different decay rates.

69. The method of Claim 34 wherein said first member is a fluorescent compound and said second members are energy donors that have excited states with different decay rates.

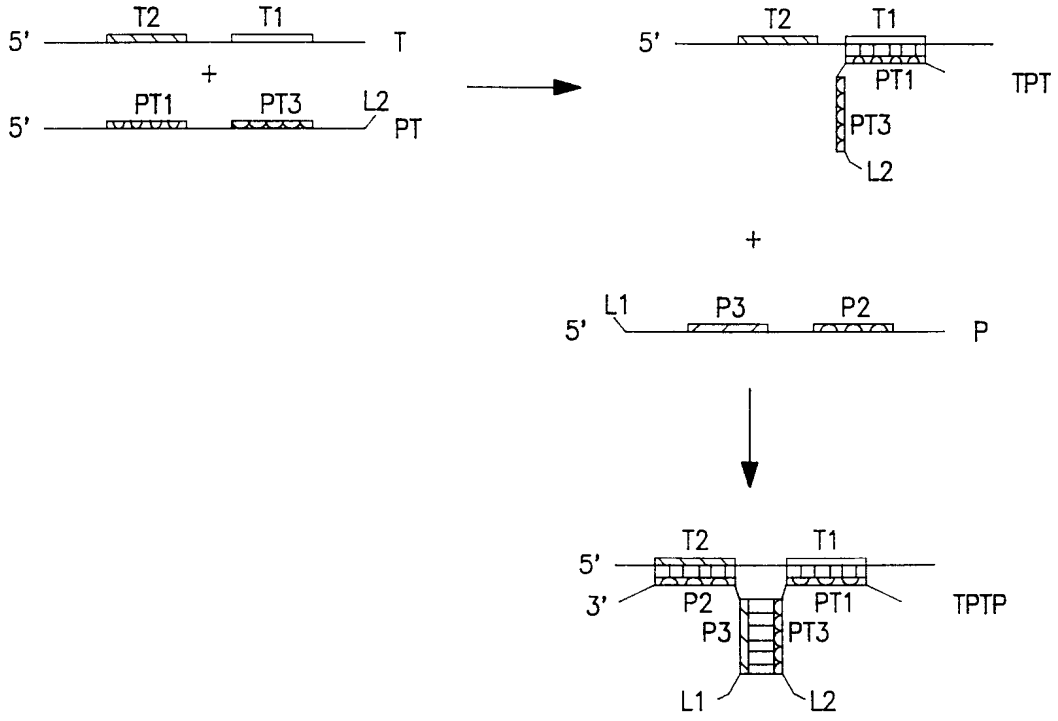

5'
T2
T1
T
+
5'
PT1
PT3
L2
PT
5'
T2
T1
PT1
TPT
PT3
L2
+
5'
L1
P3
P2
P
5'
T2
T1
3'
P2
PT1
TPTP
P3
PT3
L1
L2

FIG. 1

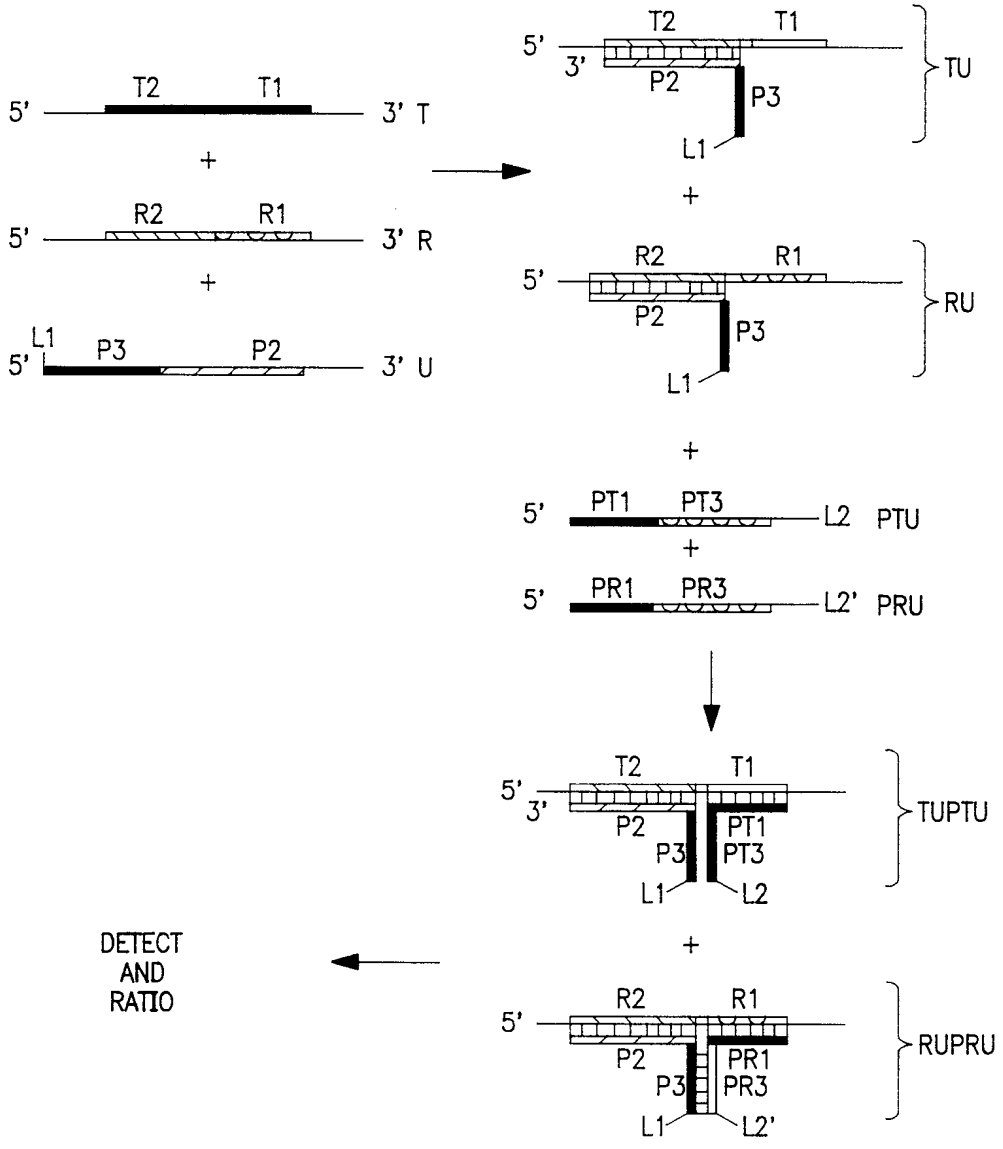

5'
T2
T1
3' T
+
5'
R2
R1
3' R
+
5'
L1
P3
P2
3' U
5'
T2
T1
3'
P2
P3
L1
TU
+
5'
R2
R1
P2
P3
L1
RU
+
5'
PT1
PT3
L2
PTU
+
5'
PR1
PR3
L2'
PRU
5'
T2
T1
3'
P2
P3
PT1
PT3
L1
L2
TUPTU
+
5'
R2
R1
P2
P3
PR1
PR3
L1
L2'
RUPRU
DETECT
AND
RATIO

FIG. 2

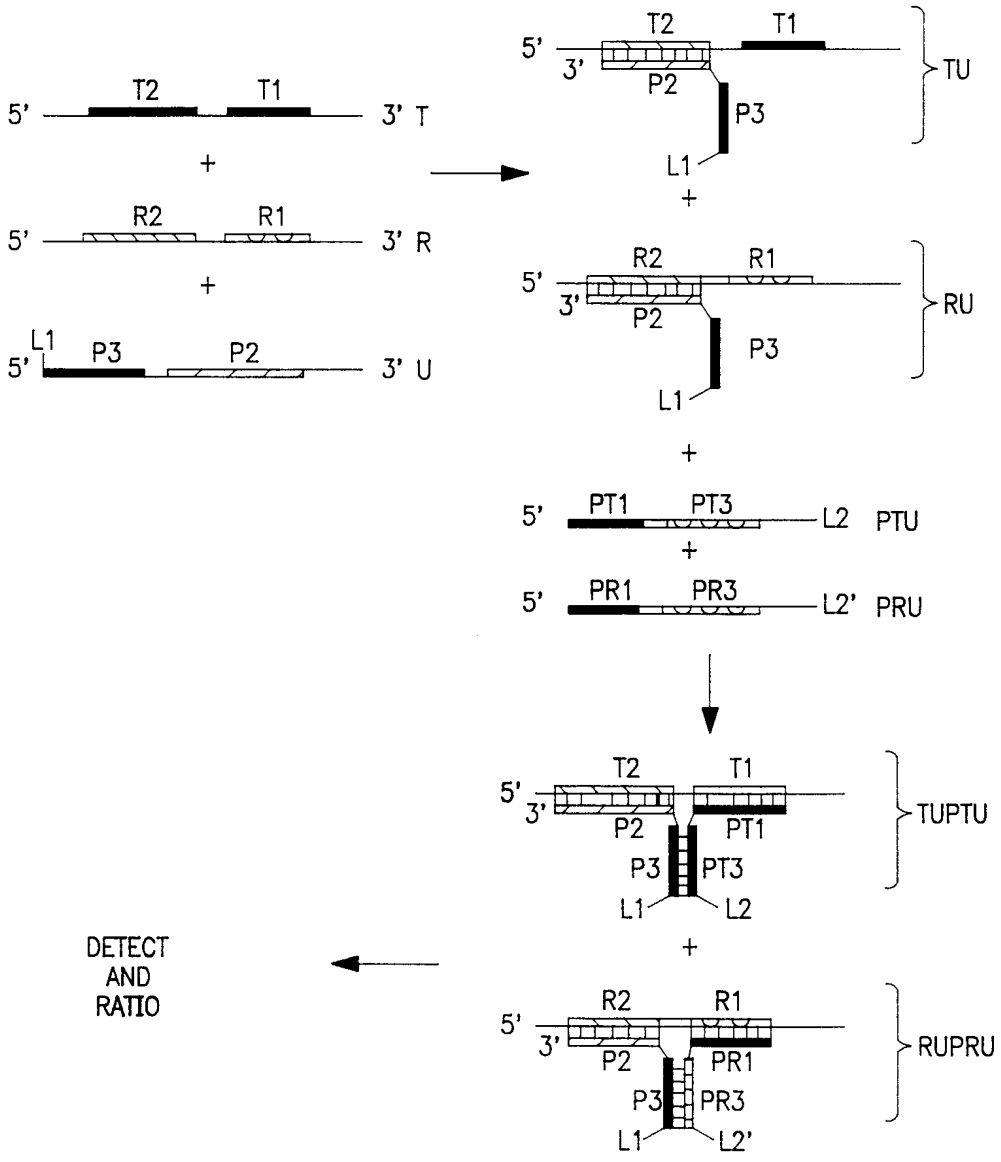

5' T2 T1 3' T
+
5' R2 R1 3' R
+
5' L1 P3 P2 3' U
5' T2 T1
3' P2
P3
L1
TU
+
5' R2 R1
3' P2
P3
L1
RU
+
5' PT1 PT3 L2 PTU
+
5' PR1 PR3 L2' PRU
5' T2 T1
3' P2 PT1
P3 PT3
L1 L2
TUPTU
+
5' R2 R1
3' P2 PR1
P3 PR3
L1 L2'
RUPRU
DETECT AND RATIO

FIG. 3

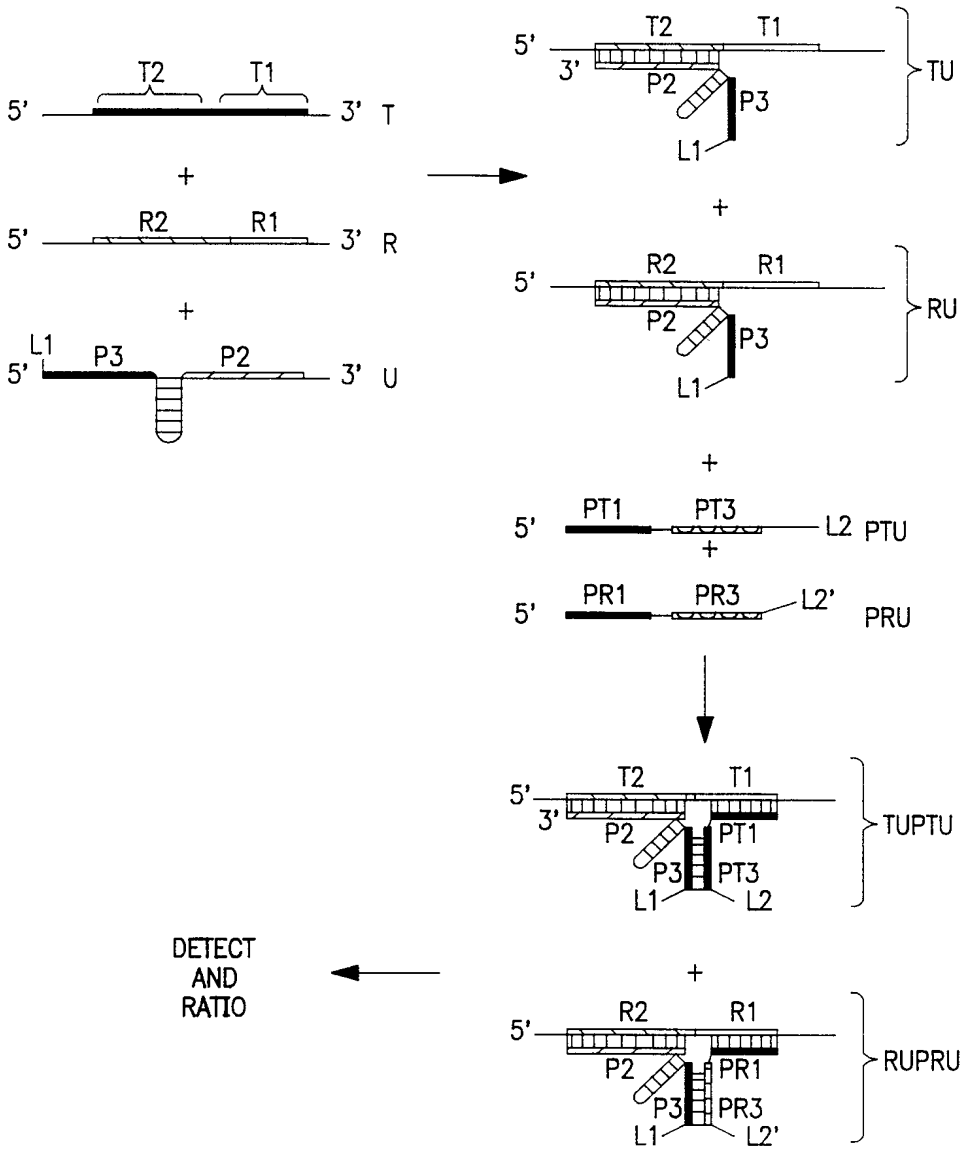

T2
T1
5'
3'
T
+
R2
R1
R
L1
P3
P2
U
P2
P3
L1
TU
RU
PT1
PT3
L2
PTU
PR1
PR3
L2'
PRU
TUPTU
RUPRU
DETECT
AND
RATIO

FIG. 4

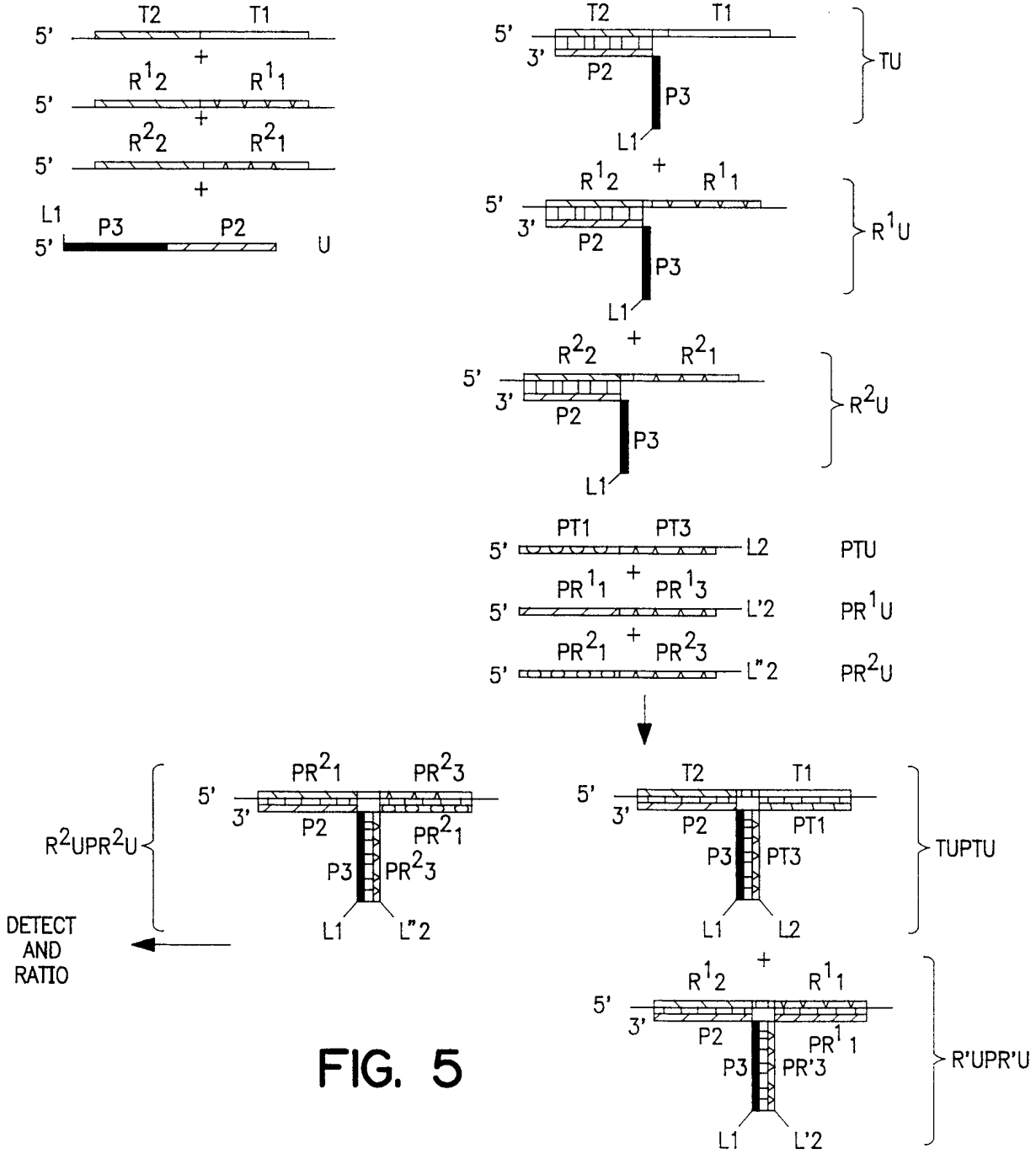

T2
T1
5'
+
R12
R11
5'
+
R22
R21
5'
+
L1
P3
P2
5'
U
3'
P2
P3
L1
TU
R1U
R2U
PT1
PT3
L2
PTU
PR11
PR13
L'2
PR1U
PR21
PR23
L"2
PR2U
R2UPR2U
DETECT
AND
RATIO
PR21
PR23
P2
PR21
P3
PR23
L"2
T2
T1
P2
PT1
P3
PT3
L2
TUPTU
R12
R11
P2
PR11
P3
PR'3
L'2
R'UPR'U

FIG. 5

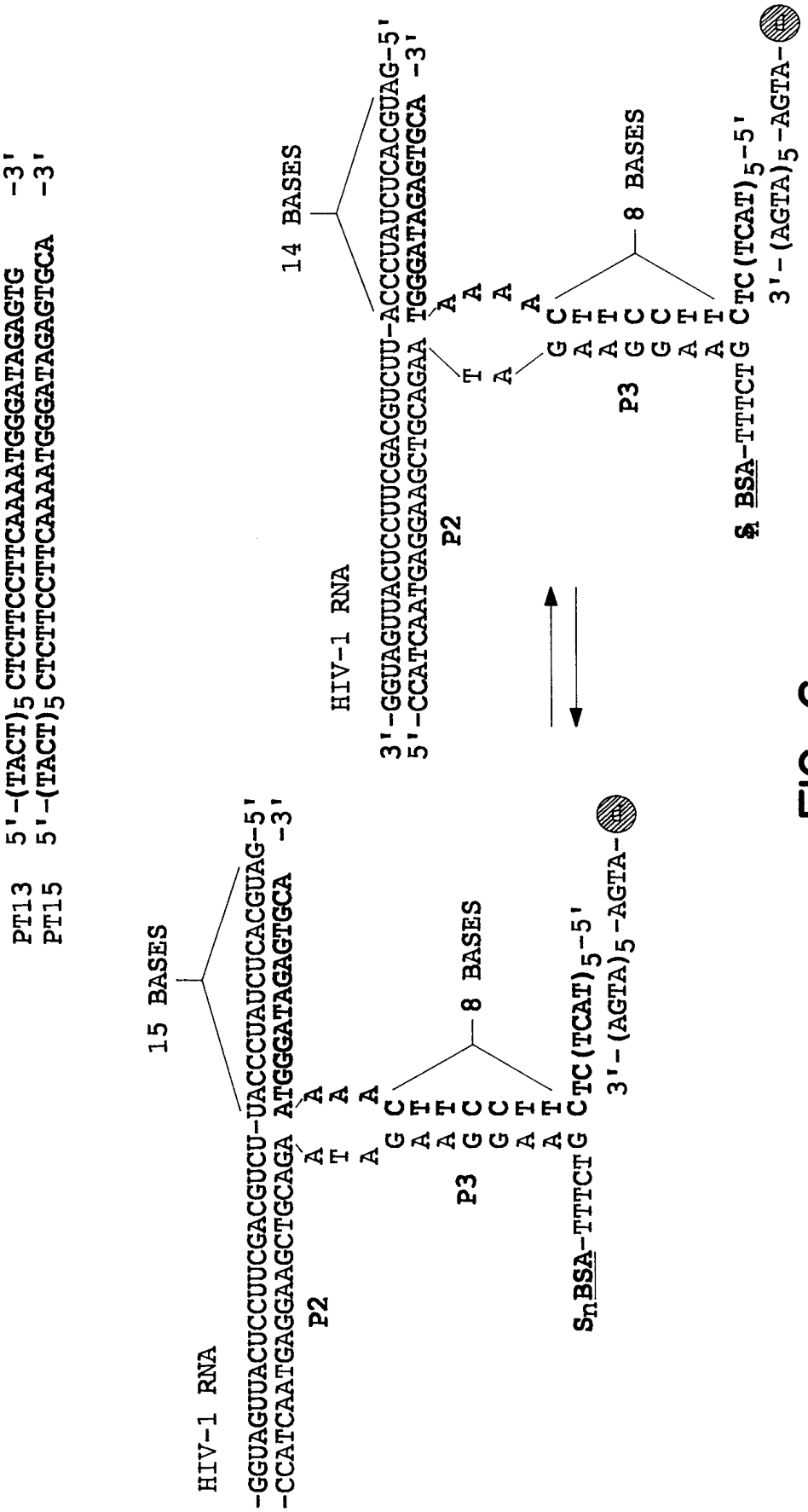

PT13 5'-(TACT)5 CTCTTCCTTCAAAATGGGATAGAGTG -3'
PT15 5'-(TACT)5 CTCTTCCTTCAAAATGGGATAGAGTGCA -3'
HIV-1 RNA
3'-GGUAGUUACUCCUUCGACGUCU-UACCCUAUCUCACGUAG-5'
5'-CCATCAATGAGGAAGCTGCAGA ATGGGATAGAGTGCA -3'
15 BASES
14 BASES
P2
P3
8 BASES
SnBSA-TTTCTG CTC(TCAT)5-5'
3'-(AGTA)5-AGTA-
BSA

FIG. 6

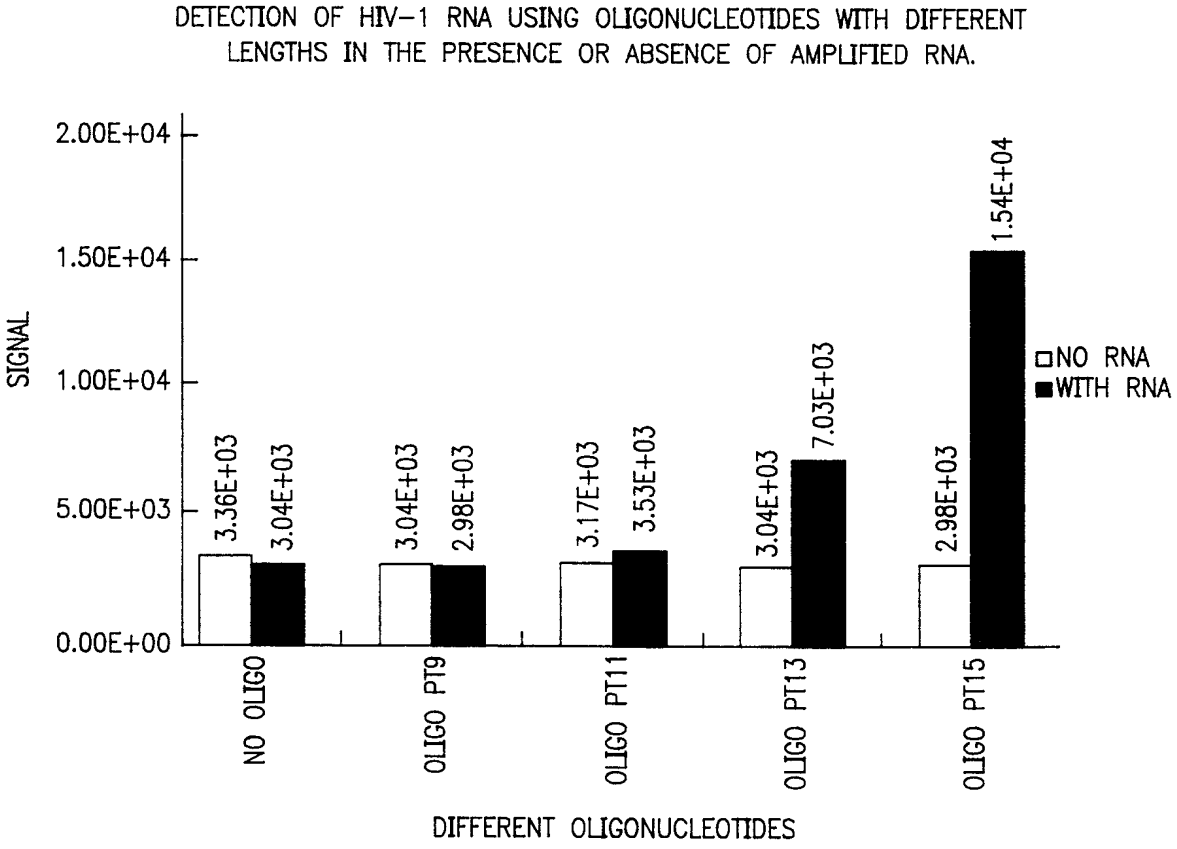

DETECTION OF HIV-1 RNA USING OLIGONUCLEOTIDES WITH DIFFERENT LENGTHS IN THE PRESENCE OR ABSENCE OF AMPLIFIED RNA.
SIGNAL
2.00E+04
1.50E+04
1.00E+04
5.00E+03
0.00E+00
3.36E+03
3.04E+03
3.04E+03
2.98E+03
3.17E+03
3.53E+03
3.04E+03
7.03E+03
2.98E+03
1.54E+04
NO RNA
WITH RNA
NO OLIGO
OLIGO PT9
OLIGO PT11
OLIGO PT13
OLIGO PT15
DIFFERENT OLIGONUCLEOTIDES

FIG. 7

INTERNATIONAL SEARCH REPORT

:national Application No
PCT/US 99/03208

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 552 931 A (GEN PROBE INC) 28 July 1993 (1993-07-28)	1-18, 23-34, 39-46, 51-59, 65-67
Y	the whole document	19-22, 35-38, 47-50, 60-64, 68,69

Y	EP 0 070 685 A (STANDARD OIL CO) 26 January 1983 (1983-01-26)	20-22, 36-38, 48-50, 61-64, 68,69
	the whole document	

	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 July 1999	04/08/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hagenmaier, S

2

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

rnational Application No
PCT/US 99/03208

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	CANTOR: "LIGHTING UP HYBRIDIZATION" BIO/TECHNOLOGY, vol. 14, 1 March 1996 (1996-03-01), page 247 XP002094958 ISSN: 0733-222X the whole document	20-22, 36-38, 48-50, 61-64, 68,69
Y	US 4 299 916 A (LITMAN DAVID J ET AL) 10 November 1981 (1981-11-10) cited in the application the whole document	19,35, 47,60
A	MORRISON ET AL.: "SOLUTION-PHASE DETECTION OF POLYNUCLEOTIDES USING INTERACTING FLUORESCENT LABELS AND COMPETITIVE HYBRIDIZATION" ANAL.BIOCHEM., vol. 183, 1989, pages 231-244, XP000444616 the whole document	
A	WO 97 07235 A (ABBOTT LAB) 27 February 1997 (1997-02-27) the whole document	
A	GIBSON U E M ET AL: "A NOVEL METHOD FOR REAL TIME QUANTITATIVE RT-PCR" GENOME RESEARCH, vol. 6, no. 10, 1 October 1996 (1996-10-01), pages 995-1001, XP000642796 ISSN: 1088-9051 the whole document	
A	GEMEN VAN B ET AL: "A ONE-TUBE QUANTITATIVE HIV-1 RNA NASBA NUCLEIC ACID AMPLIFICATION ASSAY USING ELECTROCHEMILUMINESCENT (ECL) LABELLED PROBES" JOURNAL OF VIROLOGICAL METHODS, vol. 49, 1 January 1994 (1994-01-01), pages 157-167, XP000600186 ISSN: 0166-0934 the whole document	

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

rnational Application No
PCT/US 99/03208

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0552931 A	28-07-1993	AU 665062 B	14-12-1995
		AU 3586693 A	01-09-1993
		CA 2128530 A	05-08-1993
		JP 7503139 T	06-04-1995
		WO 9315102 A	05-08-1993
		US 5451503 A	19-09-1995
		US 5424413 A	13-06-1995
EP 0070685 A	26-01-1983	CA 1190838 A	23-07-1985
		JP 1651448 C	30-03-1992
		JP 3017480 B	08-03-1991
		JP 58023795 A	12-02-1983
US 4299916 A	10-11-1981	AU 538687 B	23-08-1984
		AU 6132080 A	02-07-1981
		BR 8007330 A	30-06-1981
		CA 1138332 A	28-12-1982
		EP 0032286 A	22-07-1981
		JP 1030109 B	16-06-1989
		JP 1548194 C	09-03-1990
		JP 61269068 A	28-11-1986
		JP 1465415 C	10-11-1988
		JP 56092218 A	25-07-1981
		JP 63012260 B	18-03-1988
		US 4391904 A	05-07-1983
		US 5156953 A	20-10-1992
		US 5342759 A	30-08-1994
		US 4843000 A	27-06-1989
		US 5432057 A	11-07-1995
WO 9707235 A	27-02-1997	CA 2229226 A	27-02-1997
		EP 0845047 A	03-06-1998
		JP 11502124 T	23-02-1999